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CLCN5 5'UTR isoforms in human kidneys: differential expression analysis between controls and patients with glomerulonephritis

Monica Ceol[#], **Lisa Gianesello**[#], **Enrica Tosetto**, **Giovanna Priante**, **Dorella Del Prete**, **Franca Anglani**

Kidney Histomorphology and Molecular Biology Laboratory, Clinical Nephrology Unit, Department of Medicine-DIMED, University of Padua, Padua, Italy

[#] These authors contributed equally to this work.

Abstract

ClC-5, the electrogenic chloride/proton exchanger strongly expressed in renal proximal tubules, belongs to the endocytic macromolecular complex responsible for albumin and low-molecular-weight protein uptake. ClC-5 was found to be overexpressed in glomeruli of glomerulonephritis and in cultured human podocytes under albumin overload. The transcriptional regulation of human ClC-5 is not fully understood. Three functional promoters of various strengths and 11 different 5' untranslated region (5'UTR) isoforms of *CLCN5* messenger RNA (mRNA) were detected in the human kidney (variants 1–11). The aim of this study was to investigate the expression pattern of *CLCN5* 5'UTR variants and the *CLCN5* common translated region in glomerulonephritis. The 5'UTR ends and the translated region of *CLCN5* mRNA were analyzed using quantitative relative real-time PCR or quantitative comparative endpoint PCR with *GAPDH* as housekeeping gene in 8 normal kidneys and 12 renal biopsies from patients with glomerulonephritis. The expression profile for all variants in normal and glomerulonephritis biopsies was similar, and variant 3 and alternative variant 4 were the most abundantly expressed in both sets. In glomerulonephritis biopsies, isoforms under the control of a weak promoter (variants 4, 6 and 7) showed an increased expression leading to an increase in the *CLCN5* translated region, underscoring their importance in kidney pathophysiology. Since weak promoters can be turned on by different stimuli, these data support the hypothesis that proteinuria could be one of the stimuli capable of starting a signaling pathway that induces an increase in *CLCN5* transcription.

Correspondence to: Dr Monica Ceol, Department of Medicine-DIMED, University of Padua, Padua 35129, Italy; monica.ceol@unipd.it.

Contributors FA and ET conceived the study. MC, LG and ET conducted the experiments. MC, LG and FA designed the experiments. LG, MC and DDP analyzed the data. GP provided assistance with the experimental methodology. MC, LG, FA and DDP wrote the manuscript. All authors approved the manuscript.

Competing interests None declared.

Ethics approval All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki (latest revision 2013), and patients' data were analyzed anonymously. All patients gave their informed, written consent. The study was approved by the ethics committee for experimental studies at Padua General Hospital, protocol number 0027778 (29/05/2012).

Data availability statement Data are available upon reasonable request. Clinical data and gene expression data will be available upon reasonable request by sending an email to monica.ceol@unipd.it.

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INTRODUCTION

The electrogenic chloride/proton exchanger ClC-5 is a member of the ClC family of voltage-gated chloride channels and transporters.[1] In the kidney, ClC-5 is strongly expressed in the S3 segment of proximal tubules, where it is part of the endocytic macromolecular complex comprising megalin, cubilin, amnionless and disabled-2, which is responsible for the renal reabsorption of albumin and low-molecular-weight proteins passing the glomerular filtration barrier.[2] ClC-5 is mainly expressed on early endosomes, where it colocalizes with the V-type H^+-ATPase, prompting an efficient intraluminal acidification crucial to a proper receptor-mediated endocytotic process. ClC-5 loss of function in tubular cells causes a rare hereditary form of proximal tubulopathy known as Dent disease, which is characterized by a massive quantity of low-molecular-weight proteins in the urine.[3]

Protein loss is the hallmark of many tubular and glomerular diseases, and may be due to structural and/or functional changes in various proteins expressed in the tubular or glomerular cells.[2] In particular, albuminuria is considered a powerful independent predictor of renal disease progression, of cardiovascular disease, of death in patients with renal disease, hypertension, diabetes, or vascular disease, and also of death in the general population.[4]

Our group previously identified ClC-5 expression in human glomeruli from both normal and proteinuric kidneys, and particularly in podocytes,[5] and disclosed that it was overexpressed in diabetic nephropathy and membranous glomerulonephritis, at the messenger RNA (mRNA) and protein level.[5] We also recently demonstrated that albumin overload upregulated the expression of both the cubilin-amnionless complex, and the ClC-5 channel in human podocytes *in vitro*,[6] suggesting a role for ClC-5 in albumin endocytosis by podocytes.

The human *CLCN5* gene spans about 170 kb of genomic DNA on chromosome Xp11.22 and consists of 17 exons, including 11 coding exons (from 2 to 12). We recently reported that the 5’ untranslated region (5’UTR) of the human *CLCN5* is much more complex, however. Using RNA ligase-mediated rapid amplification of cDNA 5’ ends, we identified at least 20 exons of the *CLCN5* gene, 8 of which are in the 5’UTR region.[7] Eleven different mRNAs (5’UTR isoforms) were detected in the human kidney, as a result of the alternative splicing occurring in some of the 5’UTR exons. Two strong promoters and one weak promoter were predicted to be present in the *CLCN5* gene.[7] The isoforms under the control of the two strong promoters are variants 1, 2, 3, 8, 9, 10, and 11, while those controlled by the weak promoter are variant 4, alternative 4 containing intron 1b, variant 6 and variant 7. Open reading frame analysis suggested that all except three isoforms (variants 1, 2 and 4) code for the canonical 746 amino acid ClC-5 protein. In healthy kidney tissue, variant 3 mRNA was found to be predominantly expressed, and variant 6 and 7 mRNAs were exclusively expressed.[7] The presence of many different 5’UTR ends of *CLCN5* mRNA in the kidney underscores the complexity of this gene’s molecular structure and regulatory apparatus.

The functional significance of these regulatory regions is little understood. It has been reported that many different 5'UTR exons in a gene as well as different 5'UTR ends in a mature mRNA may serve to regulate gene expression differently in various tissues, in response to physiological and pathological stimuli, via mechanisms affecting both transcriptional and translational efficiency.[8]

The aim of this study was to investigate the expression pattern of *CLCN5* 5'UTR variants, and the *CLCN5* translated region that is common to all isoforms, in renal biopsies from controls and patients with glomerulonephritis.

MATERIALS AND METHODS

Biopsies

Two different sets of human renal biopsies were analyzed for this study: the first included control cortical tissues obtained from sites remote from the tumor-bearing renal tissue, and disclosing a normal morphology and a negative immunofluorescence; the second consisted of biopsies from patients with glomerulonephritis referred for renal biopsy for diagnostic purposes.

From 2012 to 2013, 48 patients with glomerulonephritis were referred to our unit for renal biopsy. Glomerulonephritis biopsies were obtained under ultrasound guidance using a 16-gauge needle. Only 11 control kidneys and 30 glomerulonephritis biopsies providing a sample sufficient for both standard pathological examination and molecular biology analyses were considered for the study.

Three controls and 18 glomerulonephritis kidneys were excluded from the subsequent analysis due to low RNA quantity and/or integrity (as defined and described in the next paragraph). Clinical data of 8 controls and 12 patients with glomerulonephritis enrolled in this study are provided in table 1.

In particular, the glomerulonephritis set of biopsies was composed of five lupus nephritis and seven IgA nephropathy. The two subsets did not differ in age, proteinuria, serum creatinine and hypertension. In the lupus nephritis group, female gender was predominant.

RNA extraction and cDNA synthesis

Total RNA was extracted from a small piece of biopsy material using an RNeasy Micro Kit (Qiagen, Limburg, The Netherlands) according to the manufacturer's instructions. RNA was quantified by spectrophotometric analysis using NanoDrop ND-1000 (ThermoFisher Scientific, Waltham, Massachusetts, USA). RNA quality was assessed by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technology, Santa Clara, California, USA). Only RNA with an integrity number of at least of 8 was used for PCR analyses.

Of the total RNA, 55 ng were retrotranscribed in a final volume of 20 μL. The reaction mix was prepared as follows: 5 mM $MgCl_2$; 1 mM dNTPs; 2.5 μM random hexamers; 1 U RNase inhibitor; 2.5 U MuLV reverse transcriptase (ThermoFisher Scientific) in 50 mM

KCl; 10 mM Tris HCl pH 8.3 buffer. The reaction was performed on the 2720 thermal cycler (ThermoFisher Scientific), as follows: room temperature for 10 min, 42°C for 30 min, 99°C for 5 min, and 4°C for 5 min. To exclude genomic contamination, negative control reactions were performed in parallel, omitting the reverse transcriptase.

Quantitative relative real-time PCR

Quantitative relative real-time PCR (qPCR) was performed for variants 3, 4, 6, and 7, alternative 4, and *CLCN5* translated region. For each fragment to analyze, 1.5 μL of cDNA was amplified in a 20 μL final volume of reaction mix using SYBR Green Master Mix (EurX, Gdańsk, Poland) according to the manufacturer's instructions. Reactions were performed on the Rotor Gene (Corbett Research, Qiagen) using an annealing temperature of 62°C and 0.4 μM of each primer. Melting curve analyses were used to check PCR product specificity. Amplification curves were established for all primers and resulted in efficiencies of at least 85% (see online supplementary table S1). Data were analyzed according to the ΔΔCt method, normalizing on *GAPDH* expression levels. Primer sequences were as reported elsewhere.[7]

Quantitative comparative endpoint PCR analysis

Quantitative comparative endpoint PCR analysis (RT/PCR) was performed for variants 1, 2, 8, 9, 10, and 11, which were not distinguishable by qPCR. An aliquot of 2 μL of cDNA was used to amplify these variants in a final volume of 25 μL containing 0.2 mM dNTP 0.4 μM of each primer, 0.04 U JumpStart Taq (Sigma-Aldrich, Milan Italy) in 50 mM KCl, and 10 mM Tris HCl pH 8.3. The amplification profile for each primer set consisted of an initial denaturation at 95°C for 5 min, followed by several amplification cycles (45 s at 94°C, 45 s at specific Ta °C, 1 min at 72°C), and an extension at 72°C for 7 min.[7] Primer sequences were as reported elsewhere.[7] PCR conditions are given in online supplementary table S2.

To obtain quantitative data, a kinetic strategy was applied to ascertain the appropriate number of cycles in which to quantify the RT/PCR products with the Agilent 2100 Bioanalyzer.[9] To quantify the relative expressions of the 5'UTR variants 1, 2, 8, 9, 10, and 11, a quantitative comparative analysis was conducted using *GAPDH* expression levels as internal standard.

Statistical analysis

To compare expression data between the control and glomerulonephritis biopsy sets, the results were normalized on one control sample. To analyze the variants' expression profiling in each set, data were normalized on variant 3.

Non-parametric tests were used due to the small sample size. To compare demographical and clinical data between controls and patients with glomerulonephritis, Fisher's exact test was performed for categorical variables, while Mann-Whitney U test was performed for continuous data. Mann-Whitney U test was also used to compare the expression between the two sets of biopsies. Comparisons were drawn between the variants within the same set of biopsies using Kruskal-Wallis test with Bonferroni's correction. Spearman's rank correlation test was used to analyze linear associations between variants and translated region, between

proteinuria and *CLCN5* common translated region, and between proteinuria and 5'UTR variants.

A p value <0.05 was considered statistically significant. All analyses were conducted with R software version3.5.1.[10]

RESULTS

Expression profile in control and glomerulonephritis biopsies

The distribution analysis of the *CLCN5* 5'UTR ends within each biopsy set was performed by qPCR. The expression profile did not differ substantially between normal and glomerulonephritis biopsies, for all isoforms and the *CLCN5* translated region, except for the fact that glomerulonephritis biopsies showed no significant difference between the expressions of isoforms 7 and alternative variant 4. It confirmed that, as previously reported, [7] variant 3 and alternative variant 4 were the most abundant (figure 1A,B).

The relative mRNA expression of the *CLCN5* translated region was significantly higher in the glomerulonephritis than in the control biopsies (p=0.020) (figure 2). Variants 4, 6 and 7 were also significantly more abundant in glomerulonephritis than in control biopsies (variant 4, p = 0.031; variant 6, p = 0.007; variant 7, p=0.006), alternative variant 4 showed a trend toward greater abundance (p=0.057), and variant 3 was not significantly increased (p=0.083) (figure 2).

When analyzed by RT/PCR, only variants 1 and 8 were detectable, while variants 2, 9, 10 and 11 were absent from both biopsy sets. Since they had previously been identified in a kidney library,[7] we must assume that their absence might be due to the low sensitivity of RT/PCR in amplifying large amplicons in a clinical setting.

No differences were observed comparing *CLCN5* translated region and its 5'UTR variants between IgA nephropathy and lupus nephritis subgroups.

Linear association analysis in control and glomerulonephritis biopsies

In the control biopsy set, a significant direct correlation emerged between the 5'UTR variant 4 and the *CLCN5* translated region (table 2, figure 3A).

Several significant correlations were instead detected in the glomerulonephritis biopsy set. The *CLCN5* translated region correlated strongly with variants 3 and 7, but also with variants 1 and 4 (table 2, figure 3B).

Furthermore, analyzing the expression profiles of the IgA nephropathy and lupus nephritis subgroups, we observed no linear associations among the *CLCN5* translated region and its 5'UTR variants.

In addition, no linear associations were observed with proteinuria in both glomerulonephritis biopsies set and in the IgA nephropathy or lupus nephritis subgroups.

DISCUSSION

The 5' region of the human *CLCN5* gene is complex and not entirely clear. Eleven different *CLCN5* 5'UTR ends of *CLCN5* mRNA had previously been reported.[7] The selective use of different promoters and three different start sites can affect ClC-5 expression by binding with different transcription factors and regulating translational efficiency, but the physiological and pathophysiological roles of these regulatory regions have yet to be elucidated.

One of the main pathological conditions disrupting the renal structures is protein overload, which may affect both glomerular and tubular cells.[2] It was found that ClC-5 has a role in protein handling at both the tubular and glomerular level, and it is modulated at the transcriptional level by protein overload in human podocytes *in vitro*. In the present study, we demonstrated that the *CLCN5* transcript is upregulated in the kidneys of patients with proteinuric nephropathy, thus confirming our previous findings.[56]

To shed light on the possible role of the *CLCN5* gene's 5' regulatory apparatus in response to pathological condition, we newly studied *CLCN5* 5'UTR variants in normal and glomerulonephritis kidneys. The expression profile of the 5'UTR variants was similar in the glomerulonephritis and control biopsies, presenting few differences between the two biopsy sets.

The isoforms under the control of the weak promoter containing exon 1b (ie, variants 4, 6 and 7)[7] showed a significantly stronger expression, along with an increase in the *CLCN5* transcript, in the glomerulonephritis biopsies. The significant direct correlation between the *CLCN5* translated region and variants 4 and 7 also supports the involvement of these isoforms in upregulating the *CLCN5* transcript in these biopsies. It has been demonstrated that variant 7 is kidney-specific,[7] so its higher expression could indicate a particular renal response to proteinuria-induced insult. Weak promoters can be turned on by various stimuli.[11] The different correlation pattern raised between controls and glomerulonephritis biopsies seems to suggest that proteinuria might be one of the stimuli capable of starting a signaling pathway that prompts an increase in *CLCN5* transcription. On the other hand, it is easy to imagine that glomerulonephritis *milieu* could induce a lot of molecules modulating transcription factors able to bind to the weak promoter.

The kidney-specific variant 7 encodes canonical ClC-5 protein, whereas variant 4 is believed to encode a ClC-5 protein carrying an additional inframe 20 amino acids at the NH_2 end of the amino acid chain.[7] The significance of the extended amino-terminus of the ClC-5 chain has yet to be investigated, but the different NH_2 terminal extension in the inwardly rectifying renal potassium channel comprising 19 or 26 amino acids is known to have a key role in subunit formation.[12] One may speculate that the same might be true for the ClC-5 channel that exhibits a dimeric quaternary structure in which each subunit has its own pore.[13] That said, since variant 4 is one of the less expressed isoforms in the normal kidney, could its upregulation in proteinuric states indicate the presence of a different ClC-5 protein structure? The answer to this question deserves further studies.

A comment is warranted on the alternative variant 4, which is the second most expressed 5'UTR isoform in the kidney despite it being under the control of a weak promoter. We detected an increase in alternative variant 4 in glomerulonephritis in respect to control biopsies without a statistical significance, probably due to the high variability of cases analyzed.

The alternative variant 4, first described by our group, is characterized by intron 1 retention.[9] We suggested that intron 1 might contain an internal ribosomal entry site, which provides a more efficient alternative cap-independent mechanism for regulating the initiation of translation. We also hypothesized that, in the kidney, cells might arrange a more efficient initiation of *CLCN5* transcript translation in response to particular environmental tissue conditions by modifying the expression of the alternative variant 4.

The absence of a linear association between proteinuria and *CLCN5* 5'UTR variants even if unexpected could be explained by the relatively homogeneous proteinuria levels among patients. In fact, only one patient presented nephrotic range proteinuria, while in the others proteinuria ranged between 0.38 and 2.80 g/day.

Collectively, these results lead us to hypothesize that *CLCN5* transcript isoforms are modulated by physiopathological stimuli such as proteinuria, leading to an overall enhancement of *CLCN5* transcripts mainly under the control of the weak promoter, the majority of which will encode the canonical ClC-5 protein.

The limitation of this study lies in its retrospective nature and in the small number of biopsies considered. Nevertheless, the results that we obtained with this study confirm what we previously found *in vivo* in human proteinuric nephropathies,[5] making us confident on the goodness of our data.

CONCLUSION

We know that the 5'UTR region can have several roles in translational efficiency and in inhibiting translation, probably through interaction with ribosomes and specific DNA binding proteins, or via some elements contained in non-coding regions. Even if we evaluated a small cohort of patients, we identified different levels of *CLCN5* 5'UTR isoform expression in glomerulonephritis biopsies. Further studies on ClC-5 protein expression will be needed to confirm the observed increase in *CLCN5* transcript, however, since it has already been reported that a greater mRNA expression does not always reflect an increase in protein production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance of this study

What is already known about this subject?

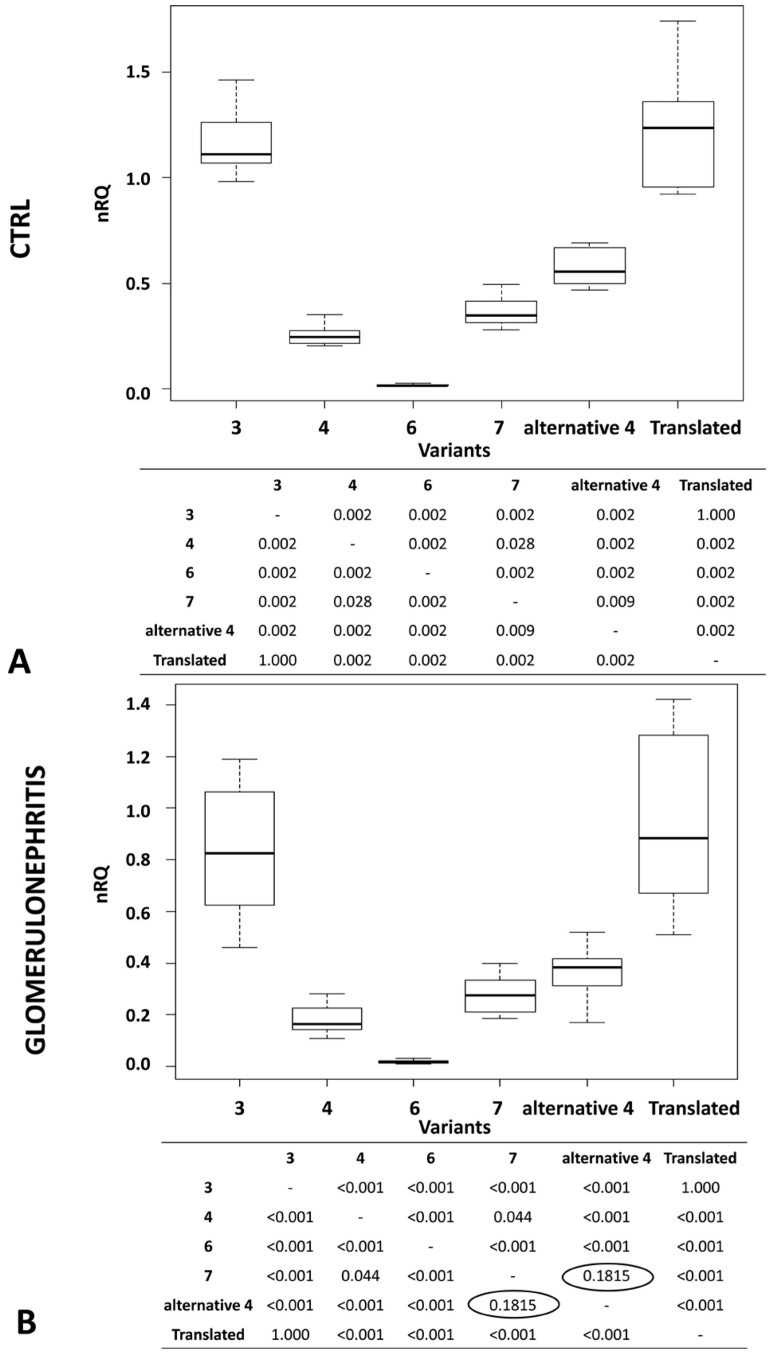
- In the human kidney, the electrogenic chloride/proton exchanger ClC-5, primarily expressed in proximal tubules, is part of the endocytic macromolecular complex involved in renal reabsorption of albumin and low-molecular-weight proteins.
- The presence of many different 5' untranslated region isoforms of *CLCN5* messenger RNA in the kidney highlights the complexity of both the molecular structure and the regulatory apparatus of the gene.
- Protein loss is the hallmark of many tubular and glomerular diseases, and albuminuria is considered a powerful independent predictor of renal disease progression, of cardiovascular disease, of death in patients with renal disease, hypertension, diabetes, or vascular disease, and also of death in the general population.

What are the new findings?

- *CLCN5* isoforms are upregulated in the kidney of patients with glomerulonephritis, especially those controlled by a weak promoter, and is kidney-specific, leading to an overall enhancement of *CLCN5* transcripts, the majority of which will encode the canonical ClC-5 protein.

How might these results change the focus of research or clinical practice?

- Since weak promoters can be turned on by various stimuli, we hypothesize that proteinuria might be one of the stimuli capable of starting a signaling pathway triggering an increase in *CLCN5* transcription.
- ClC-5 upregulation could be the right response of the organ to proteinuria, since it can prompt a more efficient protein reabsorption both at the glomerular and tubular level, thus acting as homeostatic mechanism.
- ClC-5 might be a novel target for future pathogenesis-oriented therapies for proteinuric nephropathies.



A	3	4	6	7	alternative 4	Translated
3	-	0.002	0.002	0.002	0.002	1.000
4	0.002	-	0.002	0.028	0.002	0.002
6	0.002	0.002	-	0.002	0.002	0.002
7	0.002	0.028	0.002	-	0.009	0.002
alternative 4	0.002	0.002	0.002	0.009	-	0.002
Translated	1.000	0.002	0.002	0.002	0.002	-

B	3	4	6	7	alternative 4	Translated
3	-	<0.001	<0.001	<0.001	<0.001	1.000
4	<0.001	-	<0.001	0.044	<0.001	<0.001
6	<0.001	<0.001	-	<0.001	<0.001	<0.001
7	<0.001	0.044	<0.001	-	0.1815	<0.001
alternative 4	<0.001	<0.001	<0.001	0.1815	-	<0.001
Translated	1.000	<0.001	<0.001	<0.001	<0.001	-

Figure 1.

CLCN5 expression profile. Box and whisker plots indicating *CLCN5* translated region and 5'UTR variant expression patterns in (A) control (CTRL) and (B) glomerulonephritis kidneys. P values obtained with the Kruskal-Wallis test are given under each graph. 5'UTR, 5' untranslated region, nRQ, normalized relative quantity.

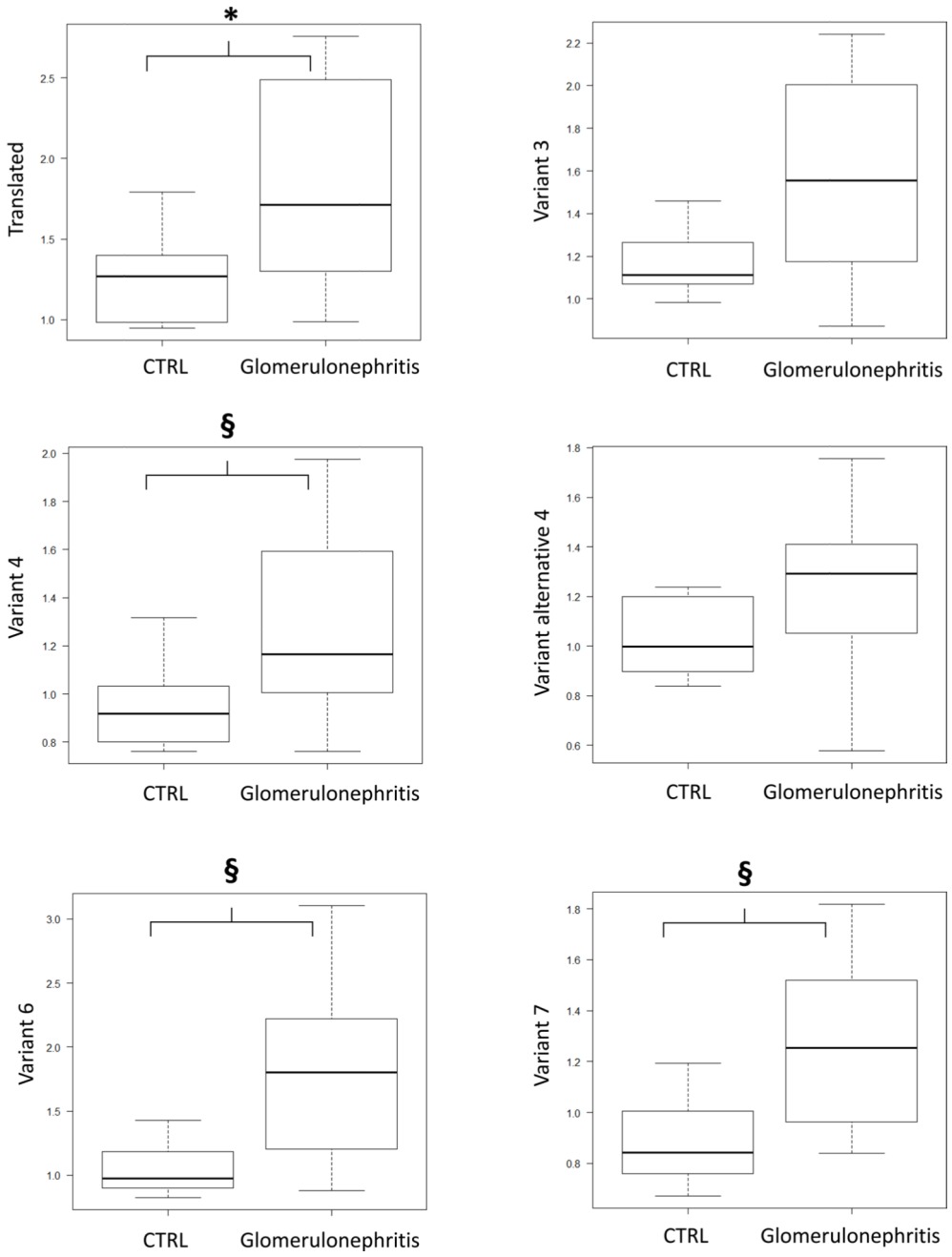


Figure 2.

Differential expression of the *CLCN5* translated region and 5'UTR ends. Box and whisker plots showing different expression levels between control (CTRL) and glomerulonephritis kidneys. Mann-Whitney U test: *p<0.05; §p<0.01. 5'UTR, 5' untranslated region.

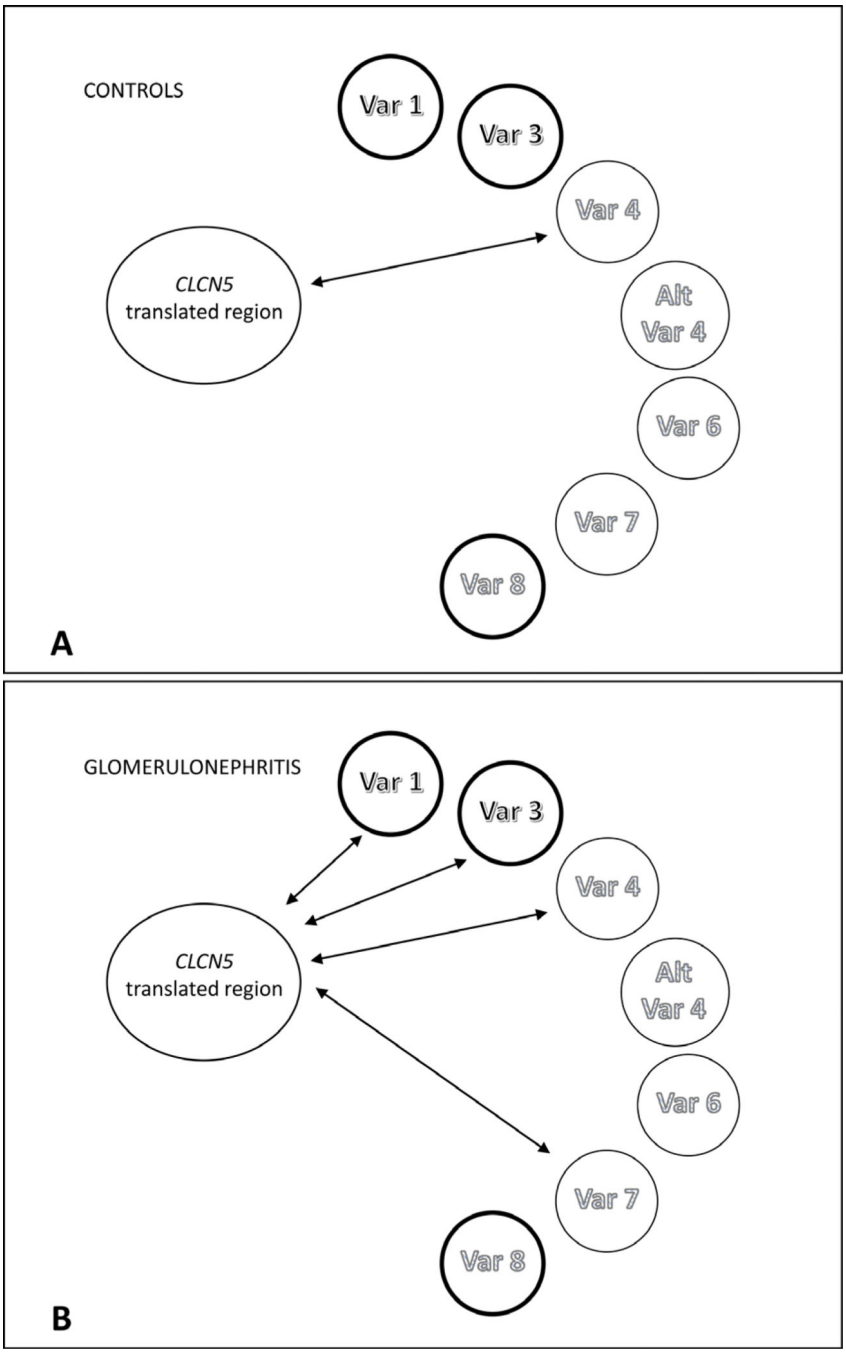


Figure 3.

Graphical representation of the linear associations between variants and *CLCN5* translated region in control (A) and glomerulonephritis (B) biopsies. Bold circles indicate the variants controlled by strong promoters.

Table 1

Demographics and clinical characteristics

	Controls (n=8)	Patients with glomerulonephritis (n=12)	P value
Age (years), median (minimum—maximum)	55 (46–71)	40 (17–57)	0.031
Gender (male), n (%)	5 (62.5)	2 (16.7)	0.041
uProt (g/day), median (minimum—maximum)	Not applicable	2.3 (0.38–6.00)	–
Serum creatinine (mg/dL), median (minimum—maximum)	1.130 (0.935–1.441)	1.025 (0.590–1.700)	0.559
Hypertension, n (%)	5 (62.5)	3 (25)	0.167

uProt, proteinuria.

Table 2

Linear association analysis in control and glomerulonephritis biopsies

Spearman's rank correlation test	**r**	**P value**
Control		
CLCN5 common translated region vs variant 4	0.833	0.015
Glomerulonephritis		
CLCN5 common translated region vs variant 1	0.670	0.017
CLCN5 common translated region vs variant 3	0.741	0.008
CLCN5 common translated region vs variant 4	0.671	0.020
CLCN5 common translated region vs variant 7	0.811	0.002