# Upregulation of Megalin, Cubilin, NGAL mRNA expression in kidney may represent tubular injury and apoptosis in chronic condition of rat diabetic model

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## ABSTRACT

**Introduction: Diabetes mellitus (DM) leads to microvascular injury development and produces diabetes nephropathy (DN) with proteinuria, tubular injury, apoptosis and autophagy with upregulation of Bax, BASP and mTORC-1. Megalin, Cubilin and Neutrophil Gelatinase Associated Lipocalin (NGAL) play role in acute pathological condition of kidney injury, however its expression in chronic and slowly progressive kidney injury such as DN has not been elucidated yet. This study focuses upregulation of Megalin, Cubilin and NGAL in association with tubular injury and apoptosis in DN condition.**

**Materials and methods: Diabetic condition was performed with intraperitoneal injection of Streptozotocin 60 mg/kg body weight (BW) in Sprague Dawley rats (2 months old, n=24), and were kept for 1, 2, and 4 months (DM1, DM2, and DM4, respectively). Control group was injected with NaCl 0.9%. Serum glucose level and proteinuria score were assessed, furthermore tubular injury score was quantified based on Periodic-Acid Schiff (PAS) staining. Reverse Transcriptase-PCR (RT-PCR) was carried out for NGAL, Megalin, Cubilin, m-TOR, Bax, and BASP-1 mRNA expression. Data were analyzed using SPSS 22 software.**

**Results: DM led to kidney injury in this model with significant higher glucose level, proteinuria and tubular injury, especially in DM4 group which represented chronic phase of DN and CKD. These findings associated with upregulation of Megalin,Cubilin and NGAL mRNA expression in DM groups, especially in DM4 group. DM4 group also revealed higher expression of Bax, BASP and mTOR mRNA expression which demonstrated apoptosis.**

**Conclusion: Megalin, Cubilin and NGAL upregulation may represent tubular injury and apoptosis as progression of DN.**

**KEYWORDS:**
*diabetes mellitus, tubular injury, Megalin/Cubilin, NGAL, apoptosis*

## INTRODUCTION

Diabetes Melitus (DM) is a chronic metabolic disease which lead to micro-vessels (microvascular) and macro-vessels (macrovascular) complications.[1] One of the microvascular complications of DM is diabetic nephropathy (DN) which becomes the high risk of End-Stage Renal Disease (ESRD).[1,2] DN is often characterized by macroalbuminuria or microalbuminuria, with targeted renal small vessel injury leading to inefficient renal function and tubular injury.[3] Detection of DN is an essential step in minimalising further complication. Elucidating signalling which represents renal injury and tubular injury severity may provide better understanding for DN mechanism.[4]

Tubular injury represents the complication of DN, furthermore upregulation of signaling from injured tubule may give early detection of DN. Neutrophil Gelatinase Associated Lipocalin (NGAL) is a transmembrane glycoprotein exclusively expressed in injured kidney and undetected in normal kidney[4], which associates with ATP depletion.[5] NGAL is also upregulated in neutrophil activation resulted from inflammation process.[4,6] Tubular injury is caused by increase reabsoption of protein in the glomerular filtrate due to filtration disruption in DN. Megalin and Cubilin are glycoproteins that may be found in various organ, including kidney, related to the endocytosis process of various protein reabsorption on epithelium.[7] Megalin plays a part in reabsorption of plasma protein leaking from glomerular filtration, meanwhile Cubilin is endocytosis receptor with molecular weight of 460 kDa that will form a ligand-receptor complex with Megalin.[8]

Apoptosis and autophagy signaling activation play role in podocyte injury, glomerulosclerosis and tubular injury.[9] Bax and Brain Acid Soluble Protein 1 (BASP1) play role in apoptosis along with p53 in intrinsic pathway,[9] and it has a direct role on renal parenchymal cell apoptosis.[10] Bax contributes directly in intrinsic pathway of apoptosis and p53 gene activation, then lead to apoptosis.[11] Other signalling such as mammalian Target of Rapamycin Complex-1 (mTORC-1) also plays an important role for autophagy and

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roles on integration process of metabolic, energy, hormonal, and nutrition stimulation to regulate cell metabolism, growth, and survival.[9,12] Physiologically, activated mTORC-1 contributes to a balanced homeostasis of β cell, adaptation, insulin secretion and cell development.[13-15] On the other hand, mTORC-1 upregulation may be caused by β-cell apoptosis.[12]

This study revealed association between upregulation of Megalin, Cubilin and NGAL with tubular injury and apoptosis in kidney of diabetic rat model, for detecting the progression of DN. Elucidating the signaling in slowly and chronic progressive kidney injury such as DN may provide underlying mechanism for biomarker of DN progression to CKD in association with renal dysfunction, apoptosis and tubular injury.

## MATERIALS AND METHODS

*Animal model of diabetes mellitus*

This research had obtained a permission from the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada with the ethical expedience number KE/FK0098/EC/2020. The DM model was performed with single intraperitoneal injection of Streptozotocin (Nacalai, 32238-91) 60mg/Kg Body Weight[16] in male Sprague Dawley rats (age 3-4 months, 150 – 200 grams, n=24). Rats were placed in cages with a light-dark cycle of 12 hours. Blood glucose level was quantified in day 5 after injection to examine the success of the model. DM was defined if the blood glucose level was higher than 200 mg/dL. Rats were divided based on the time of sacrificed, 1 month (DM1 group, n=6), 2 months (DM2 groups, n=6), 4 months (DM4 group, n=6) since we aimed to assess the progressivity of diabetes mellitus. Control group was injected with NaCl 0.9% for single dose, then sacrificed after 4 months.

For termination and sacrifice, rats were anesthetized using ketamine at a dose of 60-100mg/kg BW intramuscularly (i.m.). The blood was withdrawn from retro-orbital sinus for glucose level examination. Then, the abdomen and thorax were opened after deep anesthetized, left ventricle was perfused with NaCl 0.9%. Kidneys were harvested kept in Normal Buffer Formalin for paraffin making and RNA preservation solution for RNA extraction.

*Proteinuria score assessment*

Before termination of the rats, proteinuria score was measured with a dipstick (3 GPH strip; Uriscan®) into the urine. The score was assessed based on the positive levels in the dipstick.

*Tubular injury score assessment*

Periodic-Acid Schiff (PAS) staining was performed to examine renal histology and tubular injury score quantification. The tubular injury scores were determined through a semiquantitative scoring system. Fifteen random fields with 400x magnification were examined for each kidney, and the lesions were graded from 0 to 4 (0, no change; 1, changes affecting <25% of the section; 2, changes affecting 25 to 50% of the section; and 3, changes affecting 50 to 75%; and 4, changes affecting more than 75%), according to the area with tubulointerstitial lesions (tubular atrophy, tubular dilatation, loss of brush-border intraluminal casts, interstitial inflammation and fibrosis). The score index of each rat was expressed as a mean value of all scores obtained.

*RNA extraction, cDNA making and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)*

The RNA from fat tissues were extracted using Genezol solution (GENEzol™, Cat No. GZR100) based on the manufacturer's protocol. RNA concentrations were quantified using a nanodrop. The synthesis of RNA to cDNA was done using ReverTra Ace® (Toyobo, Cat. No. TRT-101), deoxyribonucleotide triphosphate (dNTP) (Takara, Cat. No. 4030), and primary random (TAKARA, Cat No. 3801). Reverse Transcriptase-PCR (RT-PCR) was performed for these following gene:

For RT-PCR, we used Taq Master Mix (GoTaq®Green Master Mix, Cat No. M7122). PCR products were analyzed on 2% agarose gel with DNA ladder (Bioron, Germany, Cat No. 306009). Gene expression was quantified with densitometric analysis using ImageJ software and ß-actin used to normalize expression.

## RESULTS

*DM associated with proteinuria and tubular injury*

We demonstrated that 60mg/kg BW of STZ-injection enhanced glucose levels in DM groups. The glucose level in DM1, DM2, and DM4 was significantly higher compared to the control group ($p<0.001$). Moreover, the glucose level showed significant differences among DM groups ($p<0.01$). The DM groups demonstrated disruption of renal function as shown by significantly higher proteinuria scores in DM groups compared to the control group ($p<0.001$). DM 4 group had the highest proteinuria score; however, we did not find any significant difference among DM groups. It seemed DM induced filtration disruption from the first month of induction.

**Table: The list of primers which were used in the study.**

| Gene | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) |
|---|---|---|
| Megalin | ACTGGGCAGCAGGAAATCTT | CGGGGCATATCCACTGAGAC |
| Cubilin | CTGTCCAAGGCCGTTACTGT | GATGAAAACGCCAACAGGGG |
| NGAL | CCGACACTGACTACGACCAG | CATTGGTCGGTGGGAACAGA |
| Bax | GTGAGCGGCTGCTTGTCT | GGTCCCGAAGTAGGAGAGGA |
| BASP-1 | CAAAGCCGAACTCCAAGATGGG | CGCCTTCAGCCTTCTTGTCTT |
| mTOR | CACCCATCCAACCTGATGCT | ATCGAGACCGGTAACCTCCA |
| β-actin | GCAGATGTGGATCAGCAAGC | GGTGTAAAACGCAGCTCAGTAA |

Histological observation showed tubular injury as which was characterized by tubular epithelial effacement, brush border loss, tubular lumen dilatation, tubular atrophy, and inflammatory cell invasion in DM groups. Tubular injury score quantification also revealed renal injury with significant differences among groups ($p$<0.001). The tubular injury score was higher in DM groups ($p$<0.001) compared to the control group. These results also revealed significant progression of kidney injury from the first to fourth month (from DM1 to DM4, $p$<0.001).

*DM associated with activation of Megalin-Cubilin and upregulation of NGAL*

RT-PCR analysis demonstrated DM2 and DM4 groups had significantly higher Megalin mRNA expression compared to control and DM1 groups ($p$<0.01). The upregulation of the Megalin mRNA expression was markedly observed in DM4, and there was a significant difference between DM4 and DM2 groups ($p$<0.01). Furthermore, the mRNA expression of Cubilin is clearly observed at the chronic diabetes mellitus stage. We demonstrated that DM4 represented higher Cubilin mRNA expression compared to control ($p$=0.007) and DM1 ($p$=0.049) groups. In addition, the DM4 group showed a significant upregulation of the NGAL mRNA expression ($p$=0.003) compared to the control group. We assumed that the activation of Megalin-Cubilin might associate with the progression of DM and NGAL expression.

*DM associated with upregulation of proapoptotic and autophagy pathways*

The upregulation of the BASP-1 mRNA expression was observed in DM2 and DM4 groups, not in the DM1 group. There was no significant difference in BASP-1 mRNA expression between DM1 and the control group. We demonstrated that DM2 had significance elevation compared to the control (p=0.011) group, and DM4 exhibited the highest mRNA expression of BASP-1 compared to the control (p<0.001), DM1 (p<0.001), and DM2 (p=0.001) groups.

On the other hand, the proapoptotic mRNA expression, Bax, tremendously increased in DM groups compared to the control group (p<0.001). We found that long-term hyperglycemia (DM4) enhanced the mRNA expression of Bax compared to the DM1 (p=0.026) and control (p<0.001). Besides, the mRNA expression of m-TORC1 upregulated in DM2 (p=0.006) and DM4 (p=0.037) groups compared to the control group. We found that there was no significant difference between DM2 and DM4 in the mRNA expression of m-TORC1.

## DISCUSSION

Based on the finding on this research, hyperglycemia induced renal dysfunction, apoptosis and tubular injury in association with upregulation of Megalin, Cubilin, and NGAL mRNA expression. Renal function deterioration also occurred in this study as shown by proteinuria with tubular injury. Advanced Glycation End (AGE) product in DN has broad effects on cell, tissue, chemical, and metabolic changes,[17] especially in the proximal tubules cells.[18] These cells need a high level of energy to support its role in reabsorbing a variety of molecules passing glomerular filtration and rely on aerobic metabolism in fulfilling high energy metabolic demand.[18]

Our study also revealed Megalin/Cubilin complex and NGAL mRNA upregulation might represent DM-induced tubular injury. Megalin-Cubilin-Amnion less complex in proximal convoluted tubules reabsorbs AGE in diabetic condition,[19] then stimulates AGE intoxication and the transcription factor Nuclear Factor-κB (NKκB) and Reactive Oxygen Species (ROS) activation.[17] Our study also revealed proteinuria occurred from first month of diabetic condition, however upregulation of Megalin and Cubilin occurred in 4 month of DM. Previous studies stated that Megalin expression will be increased in diabetic nephropathy, however a decreased in Megalin expression can also be found in acute condition. Proteinuria with albuminuria occurred in the first month of DM caused albumin overload which has similar effect with Megalin knockdown.[20] Megalin hyperexcretion relates with progression of DN and proximal tubules cells dysregulation in early phase of DM.[21] Cubilin functions for internalising albumin to proximal tubular epithelial cells in the normal condition. Megalin play roles in albumin reabsorption for internalising Cubilin-Albumin complex for further lysosomal degradation.[22]

Megalin/Cubilin deficiency might protect against inflammation and fibrosis.[23] Inhibition of Cubilin expression using antisense RNA delivery protects against glomerulosclerosis and tubulointerstitial injury in adryamycin treated mice.[24] Megalin positive cells upregulates inflammatory and fibrotic markers in model with anti–glomerular basement membrane antibodies injection[25] and podocyte dysfunction induction.[26] Megalin inhibition using siRNA in invitro study showed that Megalin played role in albumin-induced activation of proximal tubule synthesis of components of the renin–angiotensin system.[27] Other studies revealed upregulation of Megalin in acute kidney injury (AKI) condition. Inhibition of Megalin function has been demonstrated to ameliorate nephrotoxic acute kidney injury (AK I).[28] Megalin mediates the endogenous substances uptake, such as NGAL[29] and survivin[30] which involves in modulation and recovery from AKI.[31-33]

Upregulation of NGAL also occurs especially in the acute kidney injury (AKI) condition. Urine NGAL increased in 2 hours after injury in *Acute Kidney Injury* (AKI) condition, then reach the highest level at 6 hours, then decreased after 5 days.[34] NGAL expression which is specific in tubular epithelial cells, beside sepsis condition,[5] might provide beneficial condition as biomarker of tubular injury in chronic condition, such as DN without appearance of sepsis. Further research is needed about the relationship between Megalin expression and NGAL excreted in the urine on broader spectrum of time to ensure whether it is possible for NGAL to become a biomarker for renal injury in the future.[5] NGAL upregulation occurred primarily in acute renal pathology, furthermore it needs to study in chronic and slowly progressive CKD model with proteinuria.[23] Our study revealed Megalin, Cubilin and NGAL mRNA upregulation in chronic phase of DN as represented CKD model with proteinuria and slowly progressive mechanism. Quantification of the mRNA level is the limitation of our study, further protein level

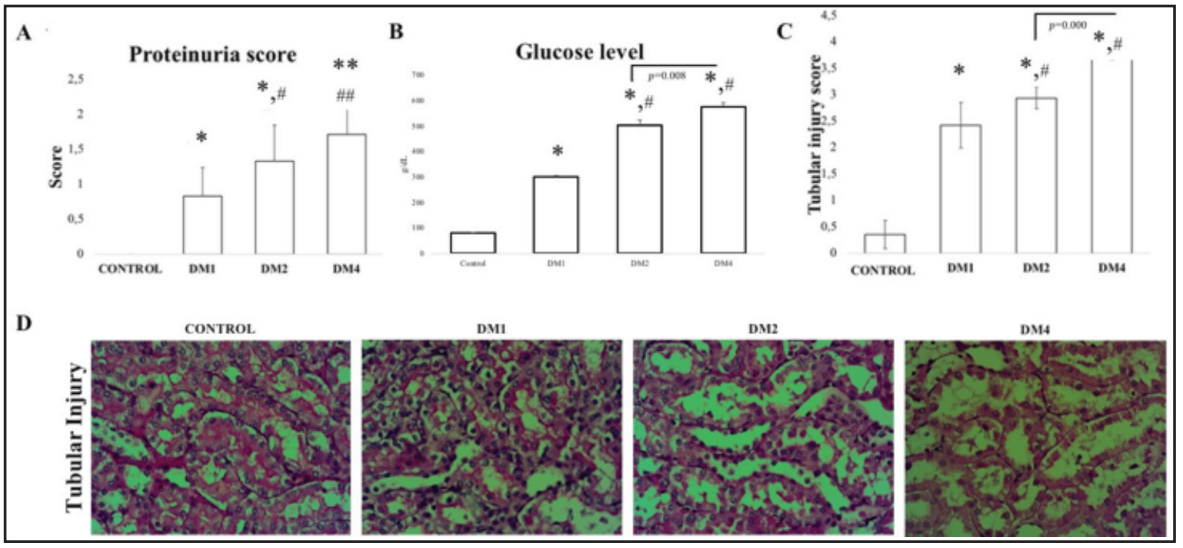


**Fig. 1:** Proteinuria and tubular injury occurred in DM groups. A. Proteinuria score quantification showed filtration disruption occurred in DM groups. B. Tubular injury score quantification showed higher tubular injury score in DM groups. C. PAS staining showed tubular injury with effacement of tubular epithelial cells and loss of brush border in tubules of DM group. *p<0.05 VS control, **p<0.01 VS control, #p<0.05 VS DM1, ##p<0.01 VS DM1.

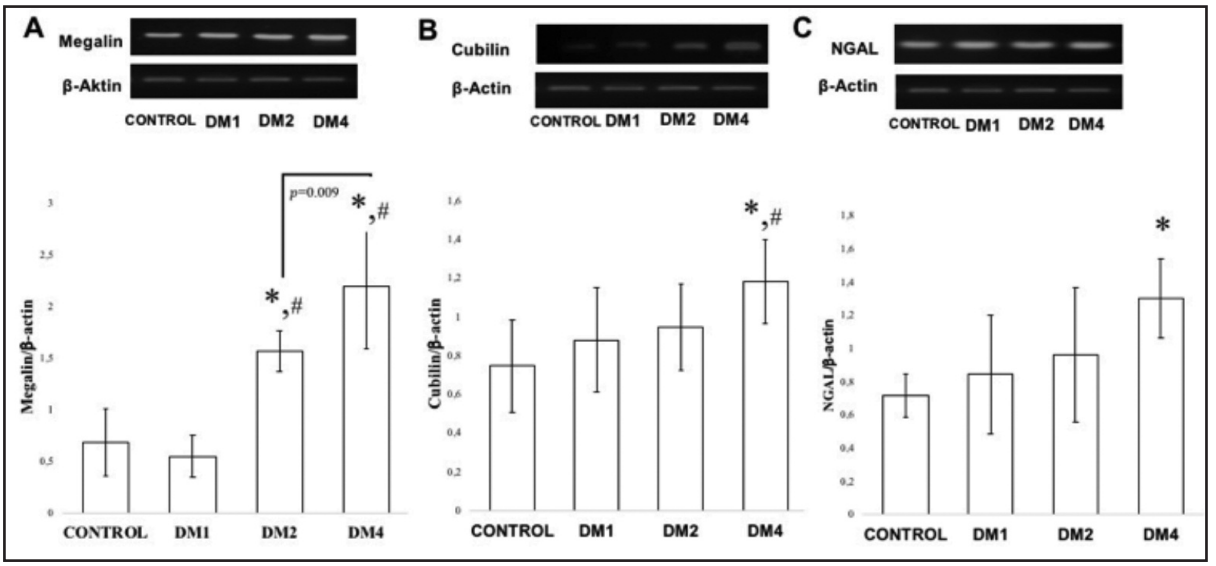


**Fig. 2:** Upregulation of Megalin, cubilin and NGAL in DM groups. A. Representative picture and densitometry analysis of Megalin mRNA expression. DM4 group demonstrated the highest Megalin mRNA expression. B. Representative picture and densitometry analysis of cubilin mRNA expression. DM4 group demonstrated the highest cubilin mRNA expression. C. Representative picture and densitometry analysis of NGAL mRNA expression. DM4 group demonstrated the highest NGAL mRNA expression. *p<0.05 VS control, **p<0.01 VS control, #p<0.05 VS DM1.

analysis of Megalin/Cubilin in tissue and urine may provide better understanding for elucidating early and chronic phase of DN, especially in association with tubular injury.

This study also demonstrated upregulation of apoptotic signaling in diabetic condition. BASP-1 is expressed by tubular cells and relates to apoptosis in DN, which leads to tubulointerstitial damage and apoptosis of tubular cells.[35] Other study also demonstrated an association between hyperglycemic state of diabetes mellitus and pro-apoptosis Bax gene expression in qualitative data in patients with type 2 diabetes mellitus.[36] In vitro study also demonstrated hyperglycemia induced apoptotic intrinsic pathway on MS-1 cell, upregulation of proapoptotic protein Bax and downregulation of antiapoptotic protein Bcl-2. It was found that treatment hyperglycemia increased Bax protein expression without changing the expression of Bcl-2.[37] Increased Bax/Bcl-2 expression ratio was obtained in diabetic nephropathy which associated with apoptosis and glomerulus size changing.[38] High glucose also induced

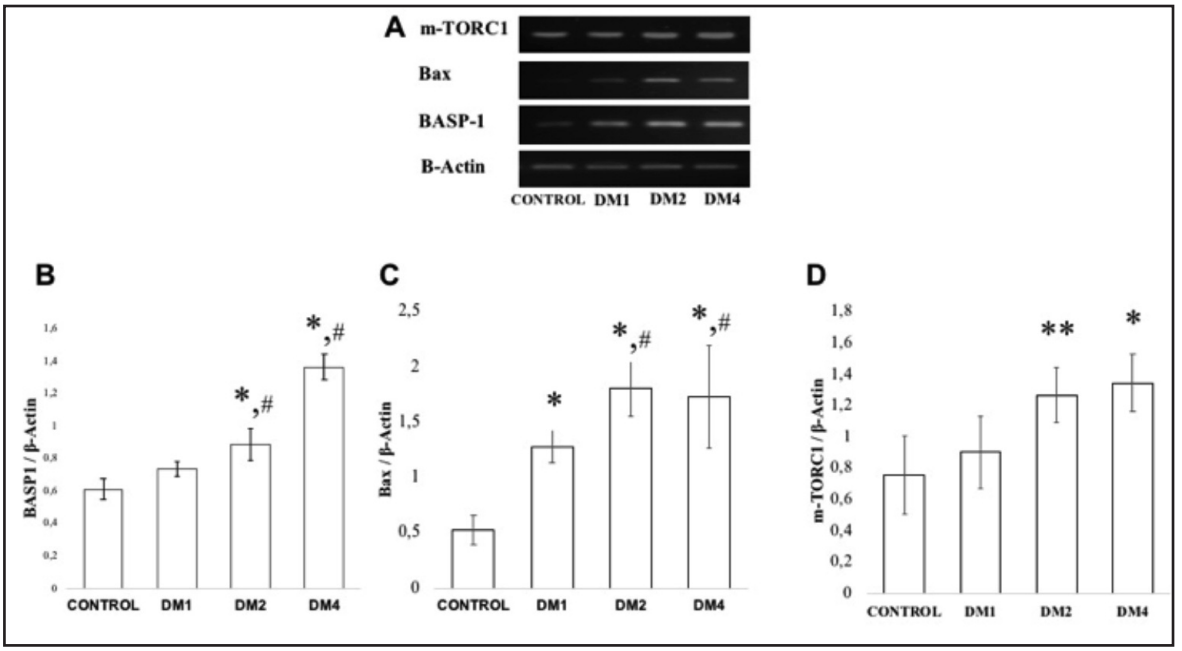


**Fig. 3:** Upregulation of BASP-1, Bax and m-TORC1 in DM groups. A. Representative picture and densitometry analysis of BASP-1 mRNA expression. DM4 group demonstrated the highest BASP-1 mRNA expression. B. Representative picture and densitometry analysis of Bax mRNA expression. DM4 group demonstrated the highest Bax mRNA expression. C. Representative picture and densitometry analysis of m-TORC1 mRNA expression. DM4 group demonstrated the highest m-TORC1 mRNA expression. *p<0.05 VS control, **p<0.01 VS control, #p<0.05 VS DM1.

apoptosis with increased on Bax/Bcl-2 expression ratio in *human embryonic kidney* (HEK) culture.[39]

Based on the research, statistically significant increased on mTORC-1, an autophagy regulator was obtained. mTORC-1 that has an anti-autophagy feature affecting impaired regeneration process in type 1 diabetes mellitus hyperglycemic model.[39] Autophagy is a cell self-degrading catabolic process needed for β cell viability, insulin secretion to stabilized blood glucose level and glucose homeostasis.[40] Activation of mTORC-1 relates to autophagy inhibition mechanism in diabetic cases. Moreover, hypertrophy of podocyte can be a predictor of renal lesion progression in patient with diabetes and mTORC-1 excessive activation in hyperglycemia condition may mediate continuous hypertrophy stimuli that can cause podocyte degeneration, glomerulosclerosis and proteinuria.[9] Elucidating protein localization of the Megalin, Cubilin and NGAL in association with apoptosis and autophagy pathway in the chronic kidney injury model, such as DN may provide better understanding for continuing this study.

## CONCLUSION

In summary, upregulation of Mgalin, Cubilin and NGAL mRNA expression may represent tubular injury and apoptosis as characteristic of DN. Elucidating protein level in kidney and urine may provide better understanding about the potency of these signaling as biomarker of DN, especially in association with tubular injury and apoptosis.

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