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Gene Expressions and Polymorphisms of Novel Biomarkers in Diabetic Nephropathy

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Authors' contributions

This work was carried out in collaboration between both authors. Author SS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author YKS managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: The prevalence of metabolic syndrome and of type 2 diabetes is extremely pronounced in Asian countries, particularly in India. In 2015, over 0.9 million deaths in India were attributed to diabetes directly or indirectly and approximately 60-70% suffering from renal diseases.
Materials and Methods: This study conducted at School of Medicine, D Y Patil University, Navi Mumbai included 241 type II diabetic patients with diabetic duration 3-5 years, between age 30-70 years. Blood samples were processed for renal parameters and RT-PCR to check the expressions and polymorphisms of IL-6, IL-10, CD 36 and LDLr.
Results: Gene analysis of IL10, CD 36 and LDLr showed that IL10, it is expressed and seen mutation at 56 A mutated to T. In CD 36 expressions were seen and amplified region showed mutation at 67 T to C (heterozygous). In LDLr both expressions and mutations of base pairs seen in higher amounts and amplified region showed mutation at 329 C to CC; 350 A to G; 352 A to G; 353 A to G; 355 A to T; 356 G to T; 358 GG to T (deletion of one nucleotide) and 563 G to T.

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Conclusion: Relationship between T2DM microalbumin and lipid parameters were not decisive and may be superseded by gene expressions and polymorphisms of IL10, CD 36, and LDLr. Early measurement of polymorphisms and expressions may prevent morbidity and mortality after therapeutic intervention and lifestyle modification. Association of cytokines may help in the development of novel biomarkers to identify individuals at risk.

Keywords: Type II diabetes; genetic predisposal; diabetic nephropathy, ESRD.

1. INTRODUCTION

Diabetes often referred as diabetes mellitus, describes a group of metabolic diseases in which the person has high blood glucose level, either because of inadequate insulin production or body's cells do not respond properly to insulin or both. This is also called hyperglycaemia. Type II diabetes mellitus (T2DM) is the most common form of diabetes all over the world. In the T2DM body does not use insulin properly. This is called insulin resistance DM. Pancrea makes extra insulin to make up for it. But, over time it isn't able to keep up and can't make enough insulin to keep blood glucose at normal levels [1]. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes.

In 2014, 9% of adults 18 years and older had diabetes [2]. Diabetes is fast gaining the status of a potential epidemic in India. More than 62 million diabetic individuals diagnosed in 2007 [3]. In 2000 India (31.7 million) topped the world with the highest number of the people with diabetes mellitus followed by China (20.8 million) with the United States (17.7 million). It was predicated that by 2030 diabetes may raise up to 79.4 million people in India following China (42.3 million) and the United States (30.3 million) [3]. Diabetes mellitus contributes greatly to morbidity, mortality, and overall health care costs. In major part, these outcomes derive from the high incidence of progressive kidney dysfunction in patients with diabetes making diabetic nephropathy a leading cause of end-stage renal disease. Three major histologic changes occur in the glomeruli of persons with diabetic nephropathy. First, mesangial expansion is directly induced by hyperglycaemia, perhaps via increased matrix production or glycation of matrix proteins.

1.1 Genetic Susceptibility

Genetically Indians are more predisposed to the development of coronary artery disease due to dyslipidaemia and low levels of high density lipoproteins compared with Caucasians (>50 yrs). Indians are more prone to development of the post DM complications at an early age (20-40 yrs) and indicate that diabetes must be carefully screened and monitored regardless of patients age within India [4]. Diabetes is a disease that is strongly associated with both micro and macrovascular complications, including retinopathy, nephropathy, neuropathy (microvascular) and ischemic heart disease, peripheral vascular disease and cerebrovascular disease (macrovascular), resulting in organ and tissue damage in approximately one-third to one-half of people in type II DM [5]. Inadequate tools are failed to recognize early detection of DN at an early stage. Serum levels of creatinine may only change after about 50-60 % of the kidney function has been lost [6]. There is utmost need to know highly sensitive biomarkers for the early detection of DN.

2. MATERIALS AND METHODS

In this study 241 subjects (118 male, 123 women, and age ranges 30-70 years) were included after screening for T2DM by measurement of blood glucose in fasting, post-prandial, glycosylated haemoglobin and microalbumin in urine. Biochemical parameters performed from fluoride plasma, glycosylated haemoglobin and gene analysis was performed from whole blood EDTA sample. Venous blood was collected in the morning after an overnight (8–12 hours) fast. Serum/plasma samples was frozen and stored at −80°C prior to analysis. All measurements were performed in a central laboratory. Categorization of subjects in three groups were done on the basis of T2DM duration 3-5 years, Glycosylated haemoglobin level (HbA1c) ≥ 7.0% and microalbuminurilt indicatesa (30-30 It indicate proximal tubular injury in diabetic conditions suggested in literature that high glucose 0 mg/dl) in study group. Blood samples were processed for other renal parameters and RT_PCR to check the expressions and polyrphisms of IL-10, CD36 and LDLr. Study protocol is approved by Institutional Ethics Committee. One Step Prime Script RT-PCR Kit (Perfect Real Time) TaKaRa

One Step Prime Script RT-PCR Kit (Perfect Real Time) is designed for Real-Time One Step RT-PCR by using Taq Man® probe1, 2 RT-PCR can be performed all in a single tube subsequently, therefore the operation is simple, and it minimizes the risk of contamination. Also, amplified products are monitored in real time, so there is no need to verify them through electrophoresis after PCR. This kit is suitable for detection of tiny amount of RNA like RNA virus. This kit uses Prime Script RTase, which has excellent extendibility and can efficiently synthesizes DNA in short time period, and TaKaRa Ex TaqHS, high efficiency hot start PCR enzyme, and they are optimized for one step RT-PCR. Combing the TaKaRa Bio RT-PCR technology with these enzymes allows this kit to have a more efficient yield of RT-PCR product (Figure 1).

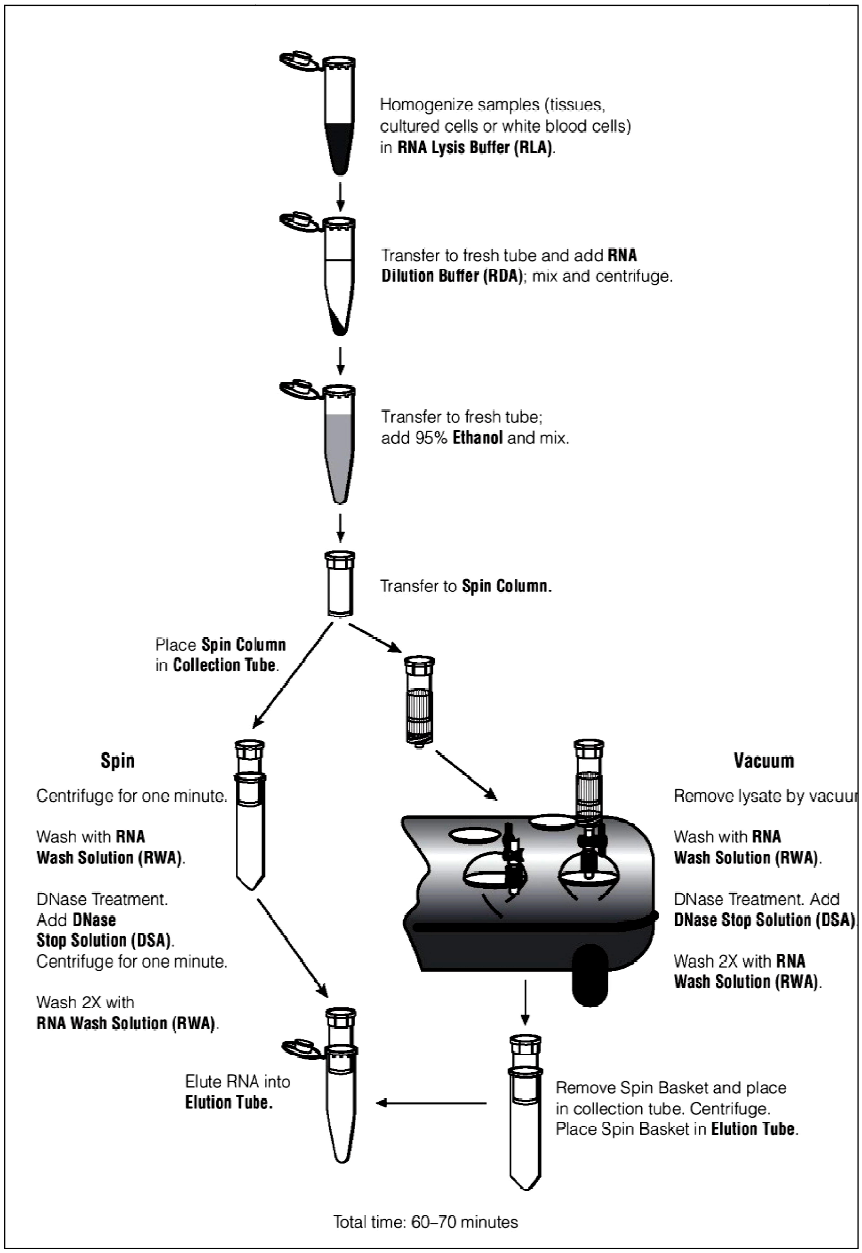

Figure 1. RNA Isolation by Prime Script RT-PCR Kit (Perfect Real Time) TaKaRa
(URL:http://www.takara-bio.com)

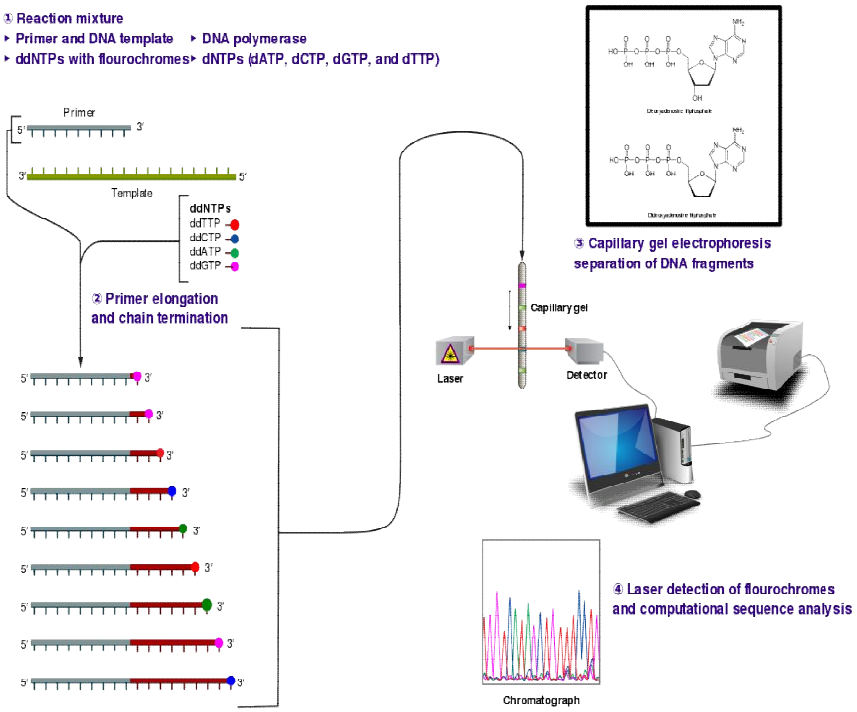

Figure 2. Gene polymorphism by Sanger sequencing method
(https://en.wikipedia.org/wiki/Sanger_sequencing)

2.1 Protocol of the Sanger Sequencing Method

The region of DNA to be sequenced is amplified and denatured to produce single stranded DNA which was further annealed to the single-stranded DNA. Dideoxynucleotide chain termination DNA sequencing then takes advantage of the fact by performing four separate reactions, each containing a DNA polymerase and a small amount of one of the four dideoxynucleotides in addition to all four deoxynucleotides, four separate sets of chain-terminated fragments can be produced. Followed by the replication/termination step, these terminated fragments bind to the single stranded DNA molecule which has acted as a template. A sequencing primer is annealed to the single-stranded DNA. The sequence of the original region of DNA is then finally deduced by examining the relative positions of the dideoxynucleotide chain termination products in the four lanes of the denaturing gel (Figure 2).

Biochemical parameters analysed by R-software for descriptive statistics which is freely available online. All sequence analysis was done using Chromas software. Sequence alignment for all samples was done using Multialign (http://multalin.toulouse.inra.fr/multalin/).

3. RESULTS

Biological data tabulated as per objectives of this study and analysed by R-software for descriptive statistics.

Table 1. Descriptive statistics for age and duration of diabetes

Parameters		N	Mean	Min	Max
Age (Yrs)	Control	80	46.26± 9.400	29.0	68.00
	30-45	80	40.01±3.892	22.0	45.00
	46-70	81	59.38±8.278	46.0	70.00
Diabetes Duration (Yrs)	Control	NA	NA	NA	NA
	30-45	80	3.49±0.729	3	6
	46-70	81	6.67±3.217	3	20

Table 2. Gender group cross tabulation

Gender population	Control	30-45 Yrs.	46-70 Yrs.	Total
Male %	41.3%	47.5%	58.0%	49.0%
Female %	58.8%	52.5%	42.0%	51.0%

Table 3. Descriptive analysis of biochemical and gene parameters between control and study groups

Parameters	Control (n=81)	< 45 years (n=80)	> 45 years (n=80)
	Mean± SD	Mean ±SD	Mean ±SD
CT IL10	26.09 1.0831	23.64 ±.6852	23.209 ±.7727
CT CD36	24.93 ±. 4925	19.48 ± 2.8954	16.1110 ±1.5848
CT LDLr	25.92±.7099	26.74 ± 1.1802	24.0746 ±1.2594
Microalbumin (<30 mg/dl)	14.14±8.49	235.28 ± 56.57	263.37 ±32.14
Creatinine (U) (20-370 mg/dl)	60.99±43.63	121.06 ± 80.65	134.57 ±129.7
A/C Ratio (30-299 mg/gm of creatinine)	0.44±0.54	3.35 ± 3.8	3.35 ±3.52
Blood Urea Nitrogen (8-20 mg/dL)	10±3	10 ±2	11 ±3
Uric Acid UA (2.6-6.0 mg/dL)	4.8±1	5.0 ± 1.8	5.3 ±1.2
Creatinine (S) (0.51-0.95 mg/dL)	.8±.2	.7 ± .2	.9 ±.2
eGFR (>/=90 ml/min/ 1.73m^2)*	100±22	114 ± 23	90 ±19

**MDRD equation: 186 x (Creatinine/88.4)$^{-1.154}$x (Age)$^{-0.203}$ x (0.742 if female) x (1.210 if black). A-albumin, C-creatinine, U-Urine and S-serum*

Table 4. Statistical analysis of gene expressions and lipid profile in study population

Dependent variable	Control (n=81)		<45 years (n=80)		>45 years (n=80)	
	<45 yrs	>45 yrs	Control	>45 yrs	Control	<45 yrs
CT OF IL10	.00	.00	.00	.00	.00	.00
CT OF CD36	.00	.00	.00	.00	.00	.00
CT OF LDLr	.00	.00	.00	.00	.00	.00
Cholesterol(T)	.070	.070	.070	.070	.070	.070
Triglyceride	.086	.086	.086	.086	.086	.086
LDL	.00	.00	.00	.00	.00	.00
HDL	.00	.00	.00	.071	.00	.071
TC /HDL ratio	0.121	.0121	.0121	.0121	.0121	.0121
LDL:HDL	.00	.00	.00	.00	.00	.00
VLDL	.086	.086	.086	.086	.086	.086

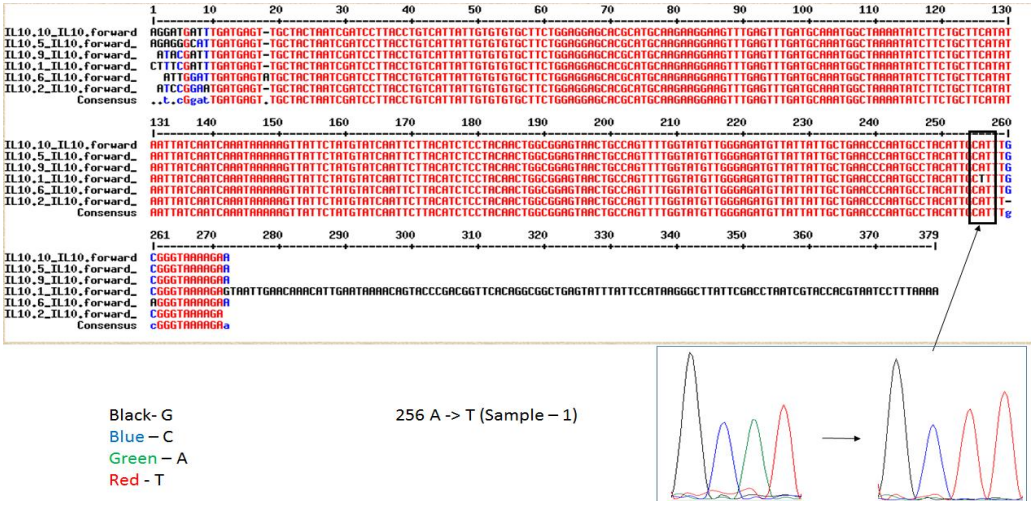

Figure 3. Genetic sequences of IL 10

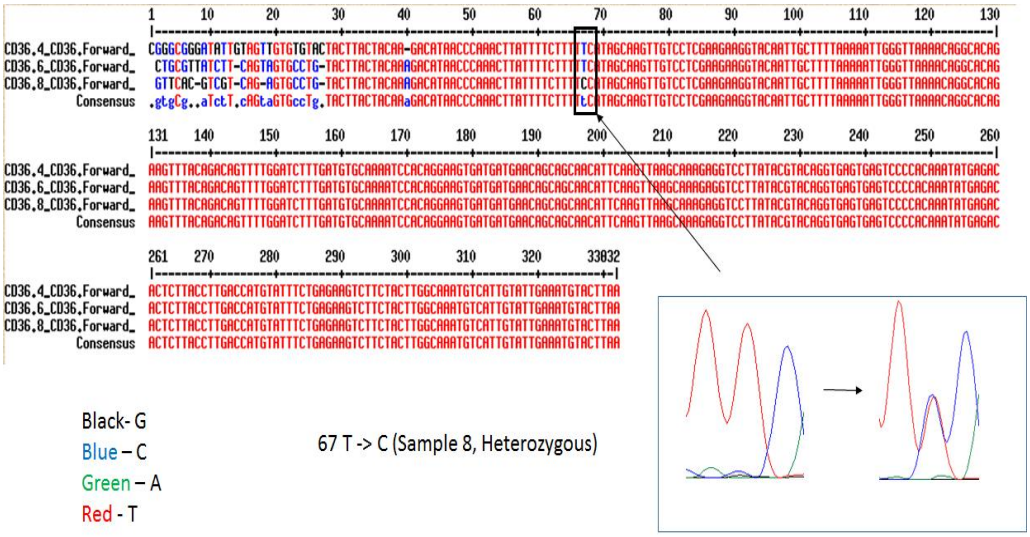

Figure 4. Genetic sequences of CD 36

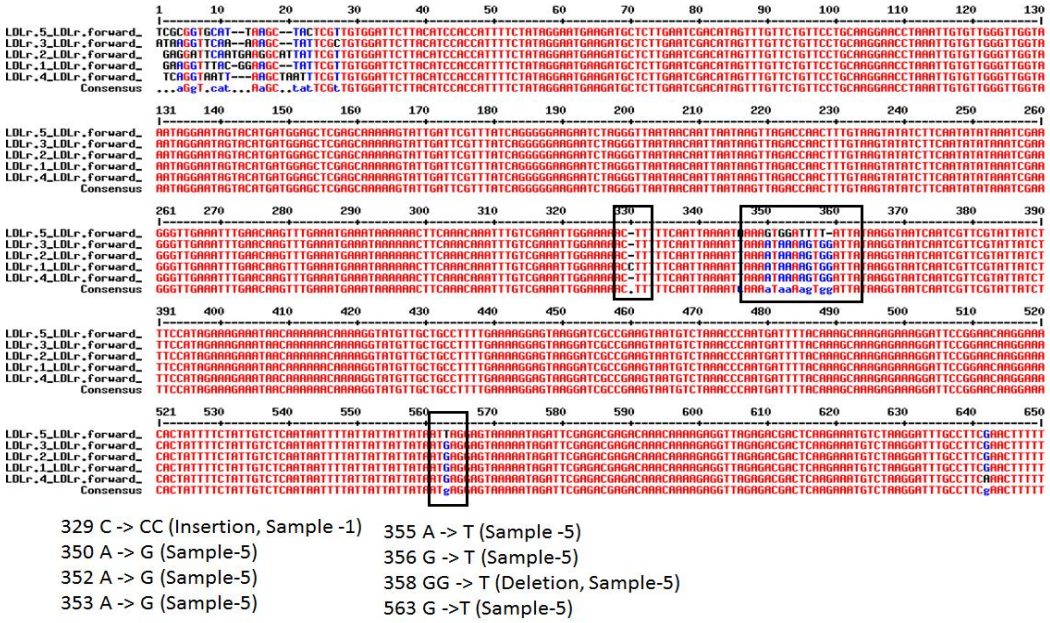

Figure 5. Genetic sequences of LDLr

4. DISCUSSION

Calculated mean age for 30-45 years groups is 40.01 with the min patient age 22 years and max patient age 45 years, while the same of 46-70 years groups is 59.38, here the minimum patient age is 46 years and maximum patient age is 70 years (Table 1).

In the gender cross tabulation, it was noted that among the 80 control patients 33 (41.3%) were male and 47 (58.8%) were female. In the 18-45 years group, 38 of 80 i.e. 47.5% were male and 42 (52.5%) were female whereas in 46-70 years group, 47 (58%) patients were male and 34 (42%) were female (Table 2).

CT of renal biomarkers and biochemical renal parameters does not manifest any concrete relationship (Table 3) It indicate that selection of subjects in study group followed inclusion and exclusion criteria strictly and unbiased. Data

analysis of gene expressions and lipid (Table 4) in study population showed significance for all three genetic parameters but non-significant to lipids except LDL and HDL.

Similar study published states that small dense LDL is correlated with the incidence and severity of DN in T2DM patients. Small dense LDL is correlated with the incidence and severity of DN in T2DM patients. [7] Author expressed that outcome of their study should be considered as a potential risk factor and as a diagnostic biomarker to be used in conjunction with other biochemical markers for early diagnosis, assessment. Similar results documented by Hirano T et al. [8] in their study high prevalence of small LDL particles in non-insulin-dependent DN. As per the study done by Inna Sinuai, et al., and Yadav KS et al. [9,10] suggested that IL-10 gene expression have an important role in the regulation and maintance of normal renal function. In this study we have noted similar findings showing expression of the IL-10 gene in the study group shows increased level of circulating IL-10 due to hyperglycaemia.

Mahfouz MH, et al. study demonstrated that the LDLr is a protective factor of CHD in Europeans. However, the case-control study showed no significant association of LDLr with CHD in Chinese population. Outcomes of this study showed that there were expressions of LDLr, it may be indicative of protective action of gene, summarized by Mahfouz MH, et al. [11]. Study done by [12] showed evidence for lipid accumulation and lipotoxicity in human kidneys. Accumulation of TGs and cholesterol in human kidney in diabetes can result from a mismatch between enhanced lipid uptake versus decreased lipid oxidation versus decreased catabolism and decreased efflux; these changes may result in alterations in lipid metabolism and lipid accumulation. They showed significant correlation between lipid metabolism, inflammation, podocyte dysfunction, fibrosis, and eGFR. Mesangial and glomerular epithelial cells express low-density lipoprotein (LDL) receptors and are capable of endocytosis of bound LDL [13]. In this study we found that triglyceride observed within reference range but LDL showed significant change. Rests of all other parameters were unaffected in all study groups. Hauden Ye et al. [14] performed meta-analysis on LDLr demonstrated that LDLr in European population is protective factor in CHD and case control study in Chinese population no correlation between LDLr and CHD. In our study qualitative estimation by photometry shows no significance but gene expression showed significant p-value.

CD36 is another trans-membrane protein of the class b scavenger receptor family and is involved in multiple biological processes. High level of glucose has been shown to induce CD36 protein synthesis in macrophages; because CD36 protein was markedly increased in proximal tubules in human DN reported by Suztac et al. [15]. In our study similar findings has been noted with significant value of expressions of CD36 in the study group compared to the controls. It was indicated that proximal tubular injury in diabetic conditions documented in literature that high glucose disrupted LDLr feedback regulation in podocytes [16], which may cause intracellular lipid accumulation and alteration of podocyte phenotype, thereby accelerating DN progression. In present study we have found similar results of LDLr gene expressions, in the study group, which might spread a light on the lipid accumulation and responsible for renal injury under diabetic condition.

Study conducted by Tarik Sqalli Houssaini et al., and Khot VV et al. [17,18], CD36 gene expression and susceptibility to nephropathies showed that expression of the CD36 gene has significant affected in the development of kidney disease. This effect is stronger when it comes from the association of two expressions in the same patient. The CD36 gene expressions in patients with hypertension, diabetes, and dyslipidaemia and/or kidney failure could even be proposed as an item in the evaluation of cardiovascular and renal risks in such patients. Polymorphism study of IL-10, CD36 and LDLr showed that IL-10, it is expressed and there is mutation at 56 A mutated to T. In CD-36 expressions were seen and amplified region showed mutation at 67 T to C (heteroxygous). In LDLr both expressions and mutations of base pairs seen in higher amounts and amplified region showed mutation at 329 C to CC; 350 A to G; 352 A to G; 353 A to G; 355 A to T; 356 G to T; 358 GG to T (delation of one nucleotide) and 563 G to T. All poltmorphic results are not enough to reach any concrete conclusion and further analysis in more number of samples is recommended (Figures 3-5).

5. CONCLUSION

It was concluded that early detection of renal injury by analyzing routine renal markers (blood urea nitrogen and serum/urine creatinine) are not

enough and decisive, which can be superseded by gene expressions and polymorphisms of IL10, CD 36, and LDLr. Early measurement of these gene polymorphisms and expressions may prevent morbidity and mortality. Prevention of CKD and awareness through education were another aspect for early intervention to limit disability and further progression of the disease. Association studies of cytokine genes will help in the development of prognostic markers to identify individuals at risk.

The prognostic regimens arising from such genetic studies will alter and ease out treatment strategies for T2DM and related complications like nephropathy. Individuals at risk will be able to take prior precautionary measures and avoid or delay the onset of the disease.

CONSENT

As per international standard written patient consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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