A clinical and in-silico analysis of hsa-miR-21 and Growth Differentiation factor-15 expression in Diabetic Nephropathy

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Abstract

Background: Diabetic Nephropathy (DN), a microvascular complication, is a major cause of end-stage renal disease (ESRD). GDF-15 and hsa-miR-21 are closely associated with endothelial dysfunction and inflammation.

Methods: In-silico analysis was used to identify GDF-15 and insulin related protein-protein interaction (PPI) network and a common set of GDF-15 regulating transcription factors. Common targeting miRNA of GDF-15 regulating transcription factors were investigated in miRNet and TargetScan. Further, 30 type 2 DN patients and 30 healthy controls were included for clinical chemistry analysis, to analyze serum GDF-15 levels by ELISA and to evaluate the fold change expression (FCE) of circulating hsa-miR-21 by RT-PCR.

Results: In the PPI network of IRS1, IRS2, INSR, IGF1R, INS, AKT1, PPARG, CEBPB, EGR1, TP53, KLF4, ATF3, GDF15, TWIST2, the common nodes between insulin and GDF-15 were identified. MicroRNA-21 was bioinformatically observed to directly target GDF-15 downregulating transcription factors KLF4, TP-53, and CEBPB. Serum GDF-15 was nearly ten (10) folds higher in DN patients ($p \leq 0.0001$) as compared to healthy controls. A positive and significant correlation of serum GDF-15 was found with HbA1c, HOMA-IR, serum urea and serum creatinine. The FCE of hsa-miR-21 was 9.18 folds higher in DN patients.

Conclusion: Raised serum GDF-15 and circulating hsa-miR-21 can serve as clinically important therapeutic targets and biomarkers of progressive renal disorder.

1. Introduction

Diabetes is a chronic metabolic disease characterized by hyperglycemia caused due to insulin insufficiency in the body. The prevalence of diabetes is highest in China and India, together accounting for nearly 180 million diabetic adults. The data is so alarming that the global prevalence of diabetes is anticipated to exceed 700 million by 2025 [1].

Type 2 Diabetes mellitus (T2DM) is a complex, multifactorial, and mostly preventable disease [2]. It can progress into micro and macro-vascular complications. One such complication isdiabetic nephropathy (DN), mainly characterized by albuminuria (≥300mg/day) and reduced glomerular filtration rate (GFR) [3].

At the molecular level, DN develops as a result of metabolic interactions, which activate intracellular signalling pathways, leading to extracellular matrix (ECM) protein accumulation, vessel permeability alteration, and proteinuria [4]. In DN, the hyper-glycaemic condition is associated with the upregulation of glucose transport-1 that also causes overexpression of transforming growth factor- β (TGF-β) in mesangial tubular cells or infiltrating renal cells [5]. This over-expressed TGF-β has been recognised as a key determinant of progressive renal function loss [6]. Furthermore, along with inducing the expression of TGF-β in mesangial cells, these factors also accelerate mesangial cell hypertrophy and ECM accumulation; as a consequence, GFR decreases and engenders chronic renal failure [6]. GDF-15, a

member of the TGF- β superfamily, has been found to be associated with T2DM. Further, an elevated serum level of GDF-15 has been reported as a clinically valuable marker for predicting progression of diabetic kidney disease (DKD) and for individual risk stratification in DN patients, with normo-albuminuria and microalbuminuria [7]. Moreover, higher levels of GDF-15 corresponds to faster deterioration of kidney function and morbidity in DN patients [8]. Hence, it is important to validate GDF-15 as an individual biomarker for the progression of DN in humans. Several findings have also highlighted that plasma GDF-15 level increases with the Mogensen stage, and is significantly correlated with microalbumin (mAlb) and eGFR, suggesting its value in early diagnosis, evaluation and prediction of the outcomes of DN [9]. A series of miRNAs, a class of short non-coding RNAs that regulate gene expression at the post-transcriptional level by binding to the 3'-untranslated regions of their target mRNAs [10], play a critical role in the regulation of many cellular and physiological activities [11]. These miRNAs either get up or downregulated in different kidney diseases [12], and can therefore serve as potential biomarkers in targeted therapy protocols [13].

In a study done on β cells, miR-21 inhibition was found to maintain the sugar level in patients with diabetic complications [5]. The expression of miR-21 is also known to be regulated by TGF-β1 and has been broadly studied in cancer biology because of its antiapoptotic effects and critical role in tumorigenesis [14]. Further, in human kidney biopsies, miR-21 was reported to increase phosphatase and tensin homolog (PTEN), and miR-21 antagonism resulted in decreased interstitial fibrosis, podocyte loss, inflammatory gene expression, albuminuria, macrophage infiltration and mesangial cell expansion [15]. In our previous review article, we have also highlighted the role of PTEN in the regulation of renal tubulointerstitial fibrosis and epithelial-mesenchymal transition (EMT) [16]. miR21 levels have also been reported to elevate with increasing disease pathology in human renal biopsies and in experimental mouse models of early/late DN [17]. However, miR-21 is regulating which member of TGF-β family, has not been investigated earlier in diabetes. Therefore, based upon previous literature we anticipated a common link between miR-21 and TGF–β family member GDF-15, therefore we investigated their interaction using bioinformatics and computational analysis, that has emerged as an efficient tool to study pathways involved in differentially expressed genes, protein-protein interaction (PPI) networks and to predict miRNA-mRNA targets [18]. Further, the expression level of serum GDF-15 was investigated in T2DM DN patients and healthy controls and was correlated with various biochemical and anthropometric parameters of the study population. Additionally, we also analysed the levels of circulating hsa-miR-21 and its correlation with serum GDF-15. Our results support the potential of a raised serum GDF-15 and circulating miR-21 to serve as therapeutic targets of progressive renal deterioration.

2. Methods

2.1 In silico investigation of the regulation of GDF-15 and its interaction with Insulin

2.1.1 Protein-Protein interaction (PPI) network

The interconnecting networks between GDF-15 and its nearest neighbours were investigated in diabetes using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) [19]. The protein IDs of insulin and GDF-15 were submitted to STRING to obtain functional interpretations of their targets in *Homo sapiens* (Figure 1). Kyoto encyclopedia of genes and genomes (KEGG) components were analysed to obtain pathway enrichment by STRING statistics taking confidence of 0.4, and the significantly enriched pathways were identified based on a value of $P < 0.05$. Plug-in of Cytoscape tool was employed [20] for cluster formation and molecular complex detection (MCODE).

2.1.2 Database Search of regulatory transcription factors of GDF 15

TRRUST transcription factors 2019, TRANSFAC and JASPAR PWMs [21], ENCODE and ChEA Consensus TFs from ChIP-X (https://genome.ucsc.edu/ENCODE/), miRNet Version: 2 [22], and Enrichr [23] were used to identify GDF-15 regulating transcription factors. The common transcription factors were selected by Venn diagram.

2.1.3 Functional annotation and enrichment analyses

The functional features of all the genes related to T2DM involved in PPI networks were analysed by two different web servers DAVID (Database for Annotation, Visualization and Integrated Discovery) [24] and STRING Version 11.0 [19]. DAVID was used for functional enrichment such as KEGG pathways and significance of gene ontology (GO) to assess the biological process, cellular components and molecular functions. Significantly enriched GO terms and KEGG pathways were selected based on $p<0.05$ and further, a false discovery rate of <0.05 to avoid false positives.

2.1.4 Construction of MicroRNA-mRNA target regulatory network

Common transcription factors were utilized to construct PPI networks, and miRNet Version: 2, a well-established miRNA target prediction database was used to predict the target miRNA of genes involved in the PPI networks [22, 25].

Considering the literature search and predicted miRs against targeted transcription factors, miR-21 was scrutinized. Further, Targetscan (http: www.targetscan.org) was used to predict the target mRNA according to miR-21 seed region. A heatmap of genes and proteins involved in PPI Networks, representing their expression in various tissues, was obtained using FunRich (Functional Enrichment analysis tool) [26].

2.2 Study population

Thirty DN patients visiting the outpatient department (OPD) of the Department of Nephrology, All India Institute of Medical Sciences (AIIMS) Jodhpur, and thirty healthy volunteers working as healthcare professionals were recruited in the study. The inclusion criteria for patients was HbA1c > 6.5% and urinary mAlb >300 mg/dl. Inclusion criteria for controls were non-diabetic subjects without any inflammatory disease or history of renal disorder.

Since GDF-15 gets upregulated due to damaged vascular endothelium, patients diagnosed with type 1 diabetes mellitus (T1DM), hypothyroidism, coronary artery disease, chronic obstructive pulmonary disease, malignancies, hepatic disorders, rheumatoid arthritis and thalassemia were excluded. In hemochromatosis, GDF-15 is secreted by erythroblasts [27], and in pregnancy, GDF-15 levels are raised by the placenta, therefore these patients were also excluded.

Venous blood samples of the study population were collected under the ethical approval of the Institutional ethics committee (IEC) of AIIMS, Jodhpur to obtain whole blood, serum and plasma. Informed consent was taken from all the participants. Urine samples were collected from the study subjects for the estimation of urinary mAlb.

2.3 Study protocol

On the same day of blood sampling and handing out of 24hr urine samples, anthropometric measurements of all the participants were recorded. Height (by a stadiometer), weight (by a weighing machine), and waist-and hip circumference (by non-elastic measuring tape) were measured. These measurements were then used to calculate body mass index (BMI) and waist-hip ratio (WHR). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) was recorded using a sphygmomanometer.

2.4 Biochemical analysis

Enzymatic colour test of human serum was performed on Beckman Coulter AU analyser using End-point method, for the quantitative determination of Fasting Blood Sugar (FBS), Low-Density Lipoprotein (LDL), High-Density Lipoprotein (HDL), cholesterol, urea and creatinine.. The Serum electrolytes sodium, potassium and chloride were analysed on the Beckman Coulter AU analyser using the Ion-Selective Electrode (ISE) principle. HbA1c was measured by latex agglutination inhibition assay. Serum insulin was analysed using the fully automated chemiluminescence analyser Diasorin Liasion. Urinary mAlb was estimated by immunoprecipitation method. The GFR of the study population was calculated using the MDRD formula GFR **(mL/min/1.73 m²)** = 175 × (serum creatinine in mg/dl) -1.154 × (Age) -0.203 × (0.742 in females).

2.5 Molecular Analysis

2.5.1 Analysis of serum GDF-15

Thermo Scientific Pierce Human GDF-15 ELISA kit was used to quantify human GDF-15 in serum, according to manufacturer's instructions. The concentration of the analyte (GDF-15) was used to obtain a standard curve. The concentration of serum GDF-15 in the samples was analysed using the standard curve by BioTek Gen5 Data analysis software.

2.5.2 Analysis of circulating hsa-miR-21

Whole blood samples of the study population were used to isolate total RNA using RBC lysis buffer in a ratio of 1:3 for Blood: RBC lysis buffer. The sample was centrifuged at 1300 rpm at 4˚C for 15 minutes and 1 ml PBS was added to dissolve the pellet, followed by centrifugation at 3000 rpm for 2 minutes. Trizol was added to the pellet (in a ratio of 10:1 for blood: trizol) to obtain a homogenous solution. Isopropanol was used to remove the aqueous layer (in a ratio of 1:1 for aqueous layer: isopropanol) and the contents were briefly vortexed. The pellet was then dissolved in 1 mL of 1% ethanol and centrifuged at 12000 x g for 5 mins. 20 µL of DEPC treated water was added after air-drying the pellet followed by incubation at 65˚C for 3-4 minutes. The sample was stored at -80˚C till further processing. Thermo-scientific NanoDrop One[c] was used to quantify the extracted total human RNA. The absorption maxima of RNA lies at 260 nm, and DEPC treated water was used as blank. An absorbance of ~2.0 at a ratio of 260 nm and 280 nm was accepted as “pure” for RNA.

2.5.3 Reverse transcription PCR

c-DNA conversion of RNA extracted, was done by Reverse Transcription PCR (RT-PCR) using Eppendorf nexus gradient Master cycler and Qiagen miScript Reverse Transcription kit as per the manufacturer’s instructions.

2.5.4 Quantification of circulating hsa-miR-21 expression by Real-Time PCR

BioRad CFX96 Real-Time System and BioRad CFX Manager Software were used for the real time expression analysis of miR21. The real time expression of miR-21 in human whole blood samples was observed using Qiagen miScript SYBR Green PCR Kit along with miScript miRNA PCR Arrays as per the manufacturer’s instructions. The fold change expression (FCE) of miR-21 in comparison to RNU6 (internal control/housekeeping gene) was evaluated using double delta/ΔΔ CT method for the cases and controls.

2.6 Statistical Analysis

IBM SPSS Statistics 21.0 for Windows (SPSS Inc, Chicago, IL) was used to analyse the skewness and kurtosis of various study parameters. Mann-Whitney U test was used to test the research hypothesis and Spearman correlation and logistic regression analysis were used to evaluate the relation between variables. Microsoft Excel software was used for graphical representation of the results.

3. Results

3.1 PPI network construction and analysis

Using STRING, GDF15 and insulin were found to interact with IRS1 (Insulin receptor substrate 1), IRS2, INSR (insulin receptor), IGF1R (insulin-like growth factor 1 receptor), AKT1 (alpha serine/threonine-protein kinase), PPARG (Peroxisome proliferator-activated receptor-gamma), CEBPB (CCAAT/enhancer-binding protein beta), EGR1 (early growth response protein 1), TP53, KLF4 (Krueppel-like factor 4), ATF3 (cyclic

AMP-dependent transcription factor ATF-3), and TWIST2 (Twist-related protein 2). Cytoscape software was used to represent the established PPI network (Figure 3).

3.2 Cluster formation by MCODE

One of the significant clusters within the PPI included INS, IGF1R, AKT1, IRS1, INSR, IRS2 and hsa-miR-21, which was selected using MCODE Score = 6.000, node- 06, edges- 15. These genes are mainly associated with the TGF-β receptor signalling and mammalian target of rapamycin (mTOR) pathway.

3.3 GO enrichment analysis

Classification of genes, involved in PPI network, into the following three classes, was used for GO analysis (Figure 2):

Cellular component class**:** the genes were enriched for the cellular components like INSR complex, protein-containing complex, nuclear transcription factor complex, receptor complex and plasma membrane protein complex.

Biological Process (BP) Class: The proteins were enriched in the cellular response to organonitrogen, peptide, response to insulin, regulation of transferase, kinase, phosphorylase activity of proteins, signal transduction, cellular response to growth factor, and signalling pathways of insulin receptor, phosphatidylinositol 3-kinase (PI3K), IGFR and mitogen-activated protein kinase (MAPK).

Molecular Function (MF) class: The proteins were enriched in the activation of INSR binding, PI3K binding, IGFR binding, RNA polymerase II regulatory region, DNA-binding transcription activator activity, RNA polymerase II-specific insulin binding, IRS binding, PI3K activity, transmembrane receptor protein tyrosine kinase activity, phosphatidylinositol-4,5-bisphosphate 3-kinase activity.

3.4 KEGG pathway enrichment analysis

PPI networks of genes were enriched (Figure 2 C) for adenosine monophosphate-activated protein kinase (AMPK) signalling pathway, forkhead box (FoxO) signalling pathway, insulin resistance, insulin signalling pathway, T2DM, mTOR signalling pathway, PI3K-AKT signalling pathway, MAPK signalling pathway, Rap1 signalling pathway, Ras signalling pathway, adipocytokine signalling pathway, adherens junction, pancreatic cancer, tyrosine kinase inhibitor resistance, AGE-RAGE signalling pathway in diabetic complications, and TNF signalling pathway.

3.5 Prediction of transcription factor and construction of miRNA regulatory networks

The target miRNAs of genes of interests were predicted by miRNet Version: 2 (an integrated platform linking miRNAs, targets & functions) and miRDB. The regulatory relationship of identified miR-21 with GDF-15 and its transcription factor was investigated by miRNet and miRDB. Cytoscape was used to construct networks based on the correlation between the target genes and their regulating transcriptome. The identified genes were ATF3, CEBPB, EGR1, KLF4, PPARG, TP53 and TWIST2, which can regulate the

levels of GDF-15. In the metabolic regulatory process of T2DM and insulin resistance, IRS1, IRS2, INSR, IGF1R, INS, AKT1, PPARG, CEBPB, EGR1, TP53, KLF4, ATF3, GDF15, TWIST2 served as important nodes in the PPI networks. MicroRNA-21 was observed as a multi-targeting miRNA as it interacted with KLF4, TP-53, and CEBPB transcription factors, which were also linked with GDF-15. Additionally, hsa-miR-21 directly targeted IGF1R. Further, the interactions also suggested a correlation between GDF-15 and miR-21, supposedly through the common transcription factors.

3.6 Demographic characteristics of the study population

In the present study, 30 DN patients and 30 healthy controls were included. Among the patients, 70% were males and 30% were females (Table 1), which reflects a higher prevalence of DN in males in the study population. This higher frequency of DN in males is predominantly due to the cross-sectional nature of the study design and does not indicate a gender-based risk of males towards DN. The age distribution of DN (Figure 2) revealed a near gaussian distribution of DN in the cases (40-80 years). The prevalence of DN in central age group (50-70 years) can be due to the fact that DN is a late onset disease, and usually advances with long standing diabetes, therefore a smaller number of patients belonged to the other age groups.

3.7 Anthropometric characteristics of the study population

A highly significant difference ($p = 0.002$) in the WHR was found between healthy controls and DN patients, wherein DN patients were found to be overweight (Table 1). DN patients also had a significantly high systolic and diastolic BP as compared to controls (Table 1). High BP of DN patients can be reasoned for hypertension (a common complication of diabetes) and a progressive cardiac myopathy condition, which is another micro-vascular complication associated with T2DM.

3.8 Clinical characteristics of the study population

The levels of FBS (184.49 ± 49.05 mg/dL), insulin (27.02 ± 28.49 µIU/mL), HbA1c levels (8.25 ± 1.70%) and HOMA-IR (12.19 ± 2.81) were found to be significantly higher in DN patients than healthy controls (Table 2). The values of GFR (96.19 ± 14.16 ml/min/1.73m^2) in the DN patients, along with highly elevated values of urea (79.31 ± 37.69 mg/dL), creatinine (2.729 ± 1.99 mg/dL) and urinary microalbumin (227.29 ± 221.21 mg/24hrs)) in the cases confirmed a comprised renal state in the DM patients.

Further, in the values of lipid profile (Cholesterol + TGL + LDL + HDL), (Table1),TGL (180.26 ± 92.23 mg/dL) level was borderline high among the DN patients. Among electrolytes (Na, K and Cl), only the value of Cl was lower than the normal (101-109 mmol/L) in DN patients.

An extremely significant difference was found in the values of FBS, insulin, HOMA-IR, urea, creatinine, Na, Cl, TGL, urinary microalbumin and GDF-15, with $p \leq 0.001$ between DN patients and healthy controls. A

highly significant difference in the value of HbA1c ($p \leq 0.05$) was also found between the groups in the study population.

3.9 Levels of GDF-15 assessed by ELISA and its correlation

The median value of serum GDF-15 was found to be nearly 10 folds higher in DN patients as compared to healthy controls (p□0.0001). The levels of GDF-15 also increased with advancement of stages of chronic kidney disease (CKD) (Figure 4C). A positive and highly significant correlation of serum GDF-15 was found with HbA1c (r = 0.747**, p = 0.000), HOMA-IR (r = 0.396*, p = 0.004), serum urea (r = 0.756*, p = 0.000) and serum creatinine (r = 0.727*, p = 0.000) (Table 2), which are the most widely accepted parameters to diagnose diabetic condition and renal disorders . The Spearman's correlation analysis of only DN cases (Table 2), revealed a positive correlation of GDF-15 with serum urea (r = 0.505*, p = 0.004) and creatinine (r = 0. 653*, p = 0.000), .

3.10 Analysis of miR-21 after quantification by RT-PCR

The FCE of circulating hsa-miR-21, assessed using RT-PCR in the study population, was found to be 9.18 folds higher in DN patients as compared to healthy controls (Table 3). Additionally, serum GDF-15 had a positive correlation withcirculating hsa-miR-21.

4. Discussion

At the molecular level, the factors that contribute to DN such as ECM protein accumulation, vessel permeability alteration, and proteinuria, develop as a result of interactions between various metabolic factors, which activate intracellular signalling pathways responsible for triggering inflammatory mediators and release of growth factors [5] like TGF-β1. The levels of TGF-β1 have been reported to increase under diabetic conditions in renal cells, including mesangial cells , hence TGF-β has been recognised as a key determinant of progressive renal function loss [6]. GDF-15, a member of the TGF-β superfamily, has been recently reported as an early marker of IR and mitochondrial dysfunction. An elevated serum level of GDF-15 was found to be associated with T2DM [7]. Several findings have also highlighted that plasma GDF-15 level increases with the Mogensen stage as an independent risk factor for increased microalbuminuria in DN patients. GDF-15 is also significantly correlated with mAlb and eGFR, suggesting its value in early diagnosis, evaluation and prediction of the outcomes of DN. Furthermore, according to our in-silico findings, and previous literature, TP53 and its family members, p63 and p73 have been reported as transcriptional regulators of GDF15, as its promoter region contains two p53-type response elements, RE1 and RE2, wherein RE2 confers p53-specific transactivation [28]. KLF4 acts as a transcriptional repressor of p53 [29]. In ovarian cancer cells, the expression of CEBPB was positively correlated with GDF15 expression and CEBPB was identified to bind with GDF15 gene promoter through luciferase reporter assay. Thus, CEBPB caused a positive regulation of GDF15 expression in cancer cells through epigenetic modification [30]. In another study, the CpG locus in MIR21 promoter was observed to be a conserved binding site of transcription factors CEBPB, MEIS3, and TEAD4, which were co-expressed with miR-21 in tumors [31]. Therefore, KLF4, CEBPB and miR-21 jointly augment EMT via

the AKT/ERK1/2 pathway by upregulating the levels of GDF-15 [32]. EMT plays an important role in renal interstitial fibrosis (RIF) with DN [33].

Our in-silico findings have also suggested the association of insulin with GDF-15 and IGF1R, which implies the role of increased GDF-15 in insulin resistance along with endothelial dysfunction, metabolic derangement and inflammation as proposed in vascular injuries and cardiovascular complications elsewhere [34]. In another study, p53 dependent expression of GDF-15 has been suggested as the link between obesity and insulin resistance, wherein activation of p53 in adipose tissue led to increased production of proinflammatory cytokines, subsequently leading to insulin resistance, and diabetes [34, 35]. Therefore, the in-silico findings of this study reiterate the connection of GDF-15 with insulin in diabetes, and along with the previous literature highlights the likelihood of GDF-15 mediated microvascular complications in DN. In a previous finding, diabetic dyslipidaemia has been reported to promote progression of DN, with few studies suggesting that TG-rich lipoprotein particles predominantly containing apolipoproteins (apos) E, C and B serve as major promoters of DN. Further, in DN patients, plasma TG levels were also suggested to be high, due to the reduced activity of hepatic lipase (HL) and lipoprotein lipase (LPL), which hydrolyze TG [35]. This is in accordance with our finding of elevated TGL levels in the patient population. The reduced serum Cl levels observed in the current study may be due to elevated serum ketoacids as observed by Yasuda K et al., wherein they have reported that in diabetics, hypochloremia can result due to elevated serum ketoacids and the ratio of Cl/ Na in DN patients was found to be significantly lower in their study as well [36].
In our molecular analysis, we found the serum levels of GDF-15 (Table 1) to be nearly ten (10) folds higher in DN patients (median 5507 pg/ml) as compared to healthy controls (567 pg/ml), ($p<0.0001$) (Figure 4A). Further, GDF-15 levels were also observed to increase with stages of DN. The highest level corresponded to Stage V (ERSD), thus confirming increasing GDF-15 to be associated with advancing stages of DN.

Spearman correlation analysis of the study population (Table 2) showed a positive correlation of serum GDF-15 with HbA1c ($r = 0.747$, $p = 0.000$), HOMA-IR ($r = 0.396$, $p = 0.004$), serum urea ($r = 0.756$, $p = 0.000$) and serum creatinine ($r = 0.727$, $p = 0.000$). Hence, serum GDF-15 levels can serve as a novel marker in indicating progression of DN and a deteriorating glycemic control in these patients. Further, the Spearman's correlation analysis of only DN cases revealed a positive correlation of GDF-15 with serum urea ($r = 0.505$, $p = 0.004$) and creatinine ($r = 0.653$, $p = 0.000$), therefore, in DN serum GDF-15 maybe a potential marker for deteriorating renal function.

The levels of miR-21 have also been reported to be higher in human renal biopsies and in experimental mouse models of early/late DN and non-DN, further, renal miR-21 knockdown showed downregulation of TGF-β1 signalling in a mouse model of T2DM [37]. The increased miR-21 expression has also been identified in the kidney biopsy samples of renal transplant patients with fibrotic kidney disease and in the urine of fibrotic patients with IgA nephropathy [38]. Since circulating miRNAs can serve as a non-invasive marker of any disease condition, Zununi and his colleagues assessed plasma samples for circulating miRNAs and observed a correlation between expression levels of miR-21 in plasma and serum creatinine

(r =-0.432, P =0.03) in renal transplant patients [39]. In another study, circulating miR-21-5p has been reported to be closely associated with endothelial dysfunction and inflammation, which are two characteristic developmentsof T2DM progression. However, there are no reports on the FCE of circulating miR-21 in relation to DN. In the present study, we observed FCE of circulating miR-21 to increase up to 9.18 folds in DN patients in comparison to healthy controls (Figure 4B). The levels of miR-21 were also found to be positively correlated with the levels of GDF-15, although the association was non-significant (which can be due to the small sample size). This positive correlation indicates the possibility of mechanistic interaction between miR-21 and GDF-15. However, GDF-15 upregulation can be reflected in any vascular endothelial damage, therefore, it can appear in diverse tissues damage responses apart from nephropathy, thus enhancing the levels of circulating GDF-15 [7]. Further, in a study done by Weronica et al., to investigate the role of GDF-15 in cardiovascular disease, GDF-15 and DNA methylation level of miR-21 promoter was found to be inversely correlated. They suggested that raised levels of GDF-15 possibly lead to demethylation of miR21 promoter, increasing its expression in cardio vascular disease [40]. However, this arena is still unexplored in DN, and a similar mechanism may be functional in DN.

5. Conclusion

This study, which is the first to the best of our knowledge, has explored the role of circulating hsa-miR-21 in DN patients in relation to GDF-15, and supports the potential of raised GDF-15 and circulating miR-21 to serve as two important signature and novel biomarkers of progressive renal deterioration. Our in silico analysis also points to a possible mechanistic regulation of GDF-15 by miR-21. Further research on the underlying mechanisms of GDF-15 and miR-21 can provide insights into an un-investigated, yet important GDF-15 and miR-21 dependent pathway, that contributes to pathogenesis of DN.

Abbreviations

AKT: alpha serine/threonine-protein kinase; BMI: Body Mass Index; ; CEBPB: CCAAT/enhancer-binding protein beta; CT: Cycle threshold; DAVID: Database for Annotation, Visualization and Integrated Discovery; DN: Diabetic Nephropathy; DBP: Diastolic Blood Pressure; ECM: Extra cellular Matrix; EGR1: Early growth response protein 1; ELISA: Enzyme Linked Immunosorbent Assay; EMT: Epithelial-mesenchymal transition; ESRD: End stage renal disease; FCE: Fold Change Expression; FunRich: Functional Enrichment analysis tool; GDF-15: Growth Differentiation Factor -15; GFR: Glomerular filtration Rate; GO: Gene Ontology; HOMA-IR: Homeostatic model assessment – Insulin Resistance; INSR: Insulin Receptor; IRS1: Insulin receptor substrate 1; ISE: Ion Selective Electrode; KEGG: Kyoto Encyclopaedia of gene and genomes; KFT: Kidney Function Test; KLF4: Krueppel-like factor 4; MCODE: molecular complex detection; miRNA: microRNA; mTOR: mammalian target of rapamycin; PI3K: phosphatidylinositol 3-kinase; PPI: Protein- Protein Interactions; PTEN: Phosphatase and tensin homolog; SBP: Systolic Blood Pressure; TGF-β1: Transforming growth factor β1; T2DM: Type 2 Diabetes Mellitus; TP53: Cellular tumour antigen p53; TWIST2: Twist-related protein 2; WHR: Waist Hip Ratio.

Declarations

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Conflict of interests:

Authors have no conflicts to report.

Data availability statement: The data will be provided when on a reasonable request.

Ethics approval:

The study was approved by AIIMS Institutional Ethics committee prior to commencement.

Consent to participate

All participants were asked for an informed consent to prior to participation, with a free will to withdraw if they wished to.

Authors contribution:

Riddhi Girdhar Agarwal- Manuscript writing, Literature review, data analysis

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Purvi Purohit- Study design, conceptualised, Manuscript writing, Literature review, data analysis

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Tables

Table 1. Comparison of different characteristics between the study population

Parameters	Healthy controls (n=30)	DN Patients (n=30)	Mann-Whitney U	Wilcoxon W	Z	P value
Anthropometric characteristics						
Age	32.83 ± 8.7	60.96 ± 11.14	48.50	513.50	-5.9	0.00*
Gender (Male %)	60	73.33	-	-	-	-
BMI (kg/m^2)	24.29 ± 2.03	26.9 ± 5.7	289	759	-2.38	0.17
WHR	0.89 ± 0.03	0.94 ± 0.06	244	699	-3.05	0.002*
Clinical characteristics						
Systolic	120.43 ± 3.7	150.00 ± 29.59	153	618	-4.4	0.000**
Diastolic	74.87 ± 2.93	83.33 ± 17.29	307.5	772.5	-2.11	0.034
Biochemical profile						
FBS (mg/dl)	94.8 ± 6.68	184.49 ± 49.05	19	484	-6.37	0.000**
2Insulin (µIU/ml)	11.9 ± 9.84	27.02 ± 28.49	304.5	769.5	-2.15	0.000**
HbA1c (%)	5.23 ± 0.26	8.25 ± 1.70	0	465	-6.66	0.031*
HOMA-IR	2.81 ± 2.38	12.19 ± 2.81	190.5	655.5	-3.83	0.000**
Urea (mg/dl)	23.03 ± 5.73	79.31 ± 37.69	25	490	-6.28	0.000**
Creatinine (mg/dl)	0.9 ± 0.16	2.729 ± 1.99	42	507	-6.03	0.000**
GFR (ml/min/1.73m^2)	96.19 ± 14.16	36.60 ± 23.05	18	483	-6.38	0.00**
Na (mmol/L)	139.56 ± 3.33	132.56 ± 5.05	70	535	-5.65	0.000**
K (mmol/L)	5.00 ± 2.16	4.69 ± 0.58	434.5	899.5	-0.22	0.819
Cl (mmol/L)	105.06 ± 2.82	99.4 ± 6.94	231	696	-3.25	0.001**
Cholesterol (mg/dl)	169.13 ±	178.16 ±	418	883	-0.47	0.636

	36.82	64.75				
TGL (mg/dl)	115.13 ± 57.84	180.26 ± 92.23	241	706	-3.09	0.002**
HDL (mg/dl)	44.3 ± 15.93	43.96 ± 11.72	432	897	-0.26	0.79
LDL (mg/dl)	106.23 ± 35.18	113.13 ± 53.99	431	896	-0.28	0.779
Urinary microalbumin (mg/24hrs)	5.49 ± 3.97	227.29 ± 221.21	58	532	-5.79	0.000**
Proteomic Profile						
Serum GDF-15	567.36 ± 377.91	5507 ± 2759.89	0	465	-6.65	0.000**

Mann-Whitney U test scores of biochemical parameters, the significantly different variables are represented by $p \leq 0.001$ = ** and $p \leq 0.05$ = *

Table 2. Spearman correlation of GDF-15 with various parameters

	Study Population (n=60)		DN patients (n=30)	
Parameters	Rho	Sig (2-tailed)	Rho	Sig (2-tailed)
FBS (mg/dl)	-0.73	0.000**	-0.18	0.316
Insulin (µIU/ml)	0.15	0.237	-0.24	0.196
HbA1c (%)	0.74	0.000**	-0.11	0.557
HOMA-IR	0.36	0.004**	-0.28	0.133
Urea (mg/dl)	0.75	0.000**	0.50	0.004**
Creatinine (mg/dl)	0.72	0.000**	0.65	0.000**
Na (mmol/L)	-0.72	0.000**	-0.34	0.065
K (mmol/L)	-0.04	0.743	0.04	0.812
Cl (mmol/L)	-0.28	0.027*	0.28	0.134
Cholesterol (mg/dl)	0.09	0.456	-0.22	0.239
TGL (mg/dl)	0.32	0.011*	-0.08	0.655
HDL (mg/dl)	0.03	0.769	-0.16	0.391
LDL (mg/dl)	0.09	0.496	-0.17	0.36
Urinary microalbumin (mg/24hrs)	0.70*	0.000**	0.30	0.106
Δct miR-21	-0.05	0.697	-0.12	0.517

A [Sig.2(tailed)] p-value ≥ 0.05 indicates a non-significant difference between the variables, p ≤ 0.05(*) indicates a significant difference between the variables and p ≤ 0.005(**) indicates an extremely significant difference.

Table 3. FCE of miR-21 in DN patients in comparison to healthy controls

Healthy Control (Δct) (n=30)	DN patients (Δct) (n=30)	ΔΔct	Fold Change Expression
2.94	-0.26	-3.20	9.18

Figures

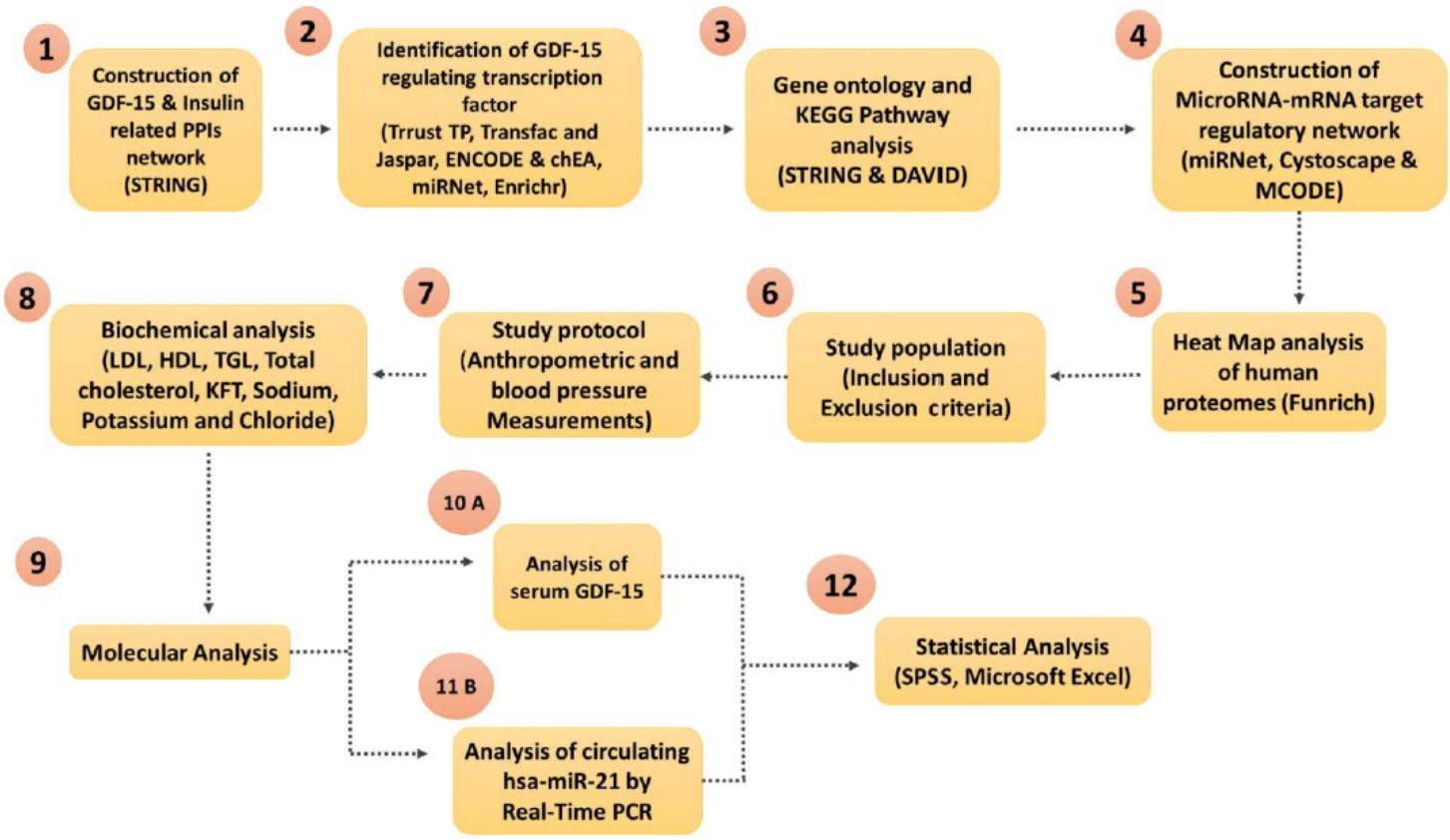

Figure 1

Flow Chart of the methodology.

Figure 2

GDF15 and Insulin related Gene Ontology enriched terms obtained from Annotation Databases; (A) Biological Process (BP); (B) Molecular Function; (C) Enriched KEGG Pathway (D) Cellular components; (E) Venn diagram of GDF15 Transcription factor of four different databases.

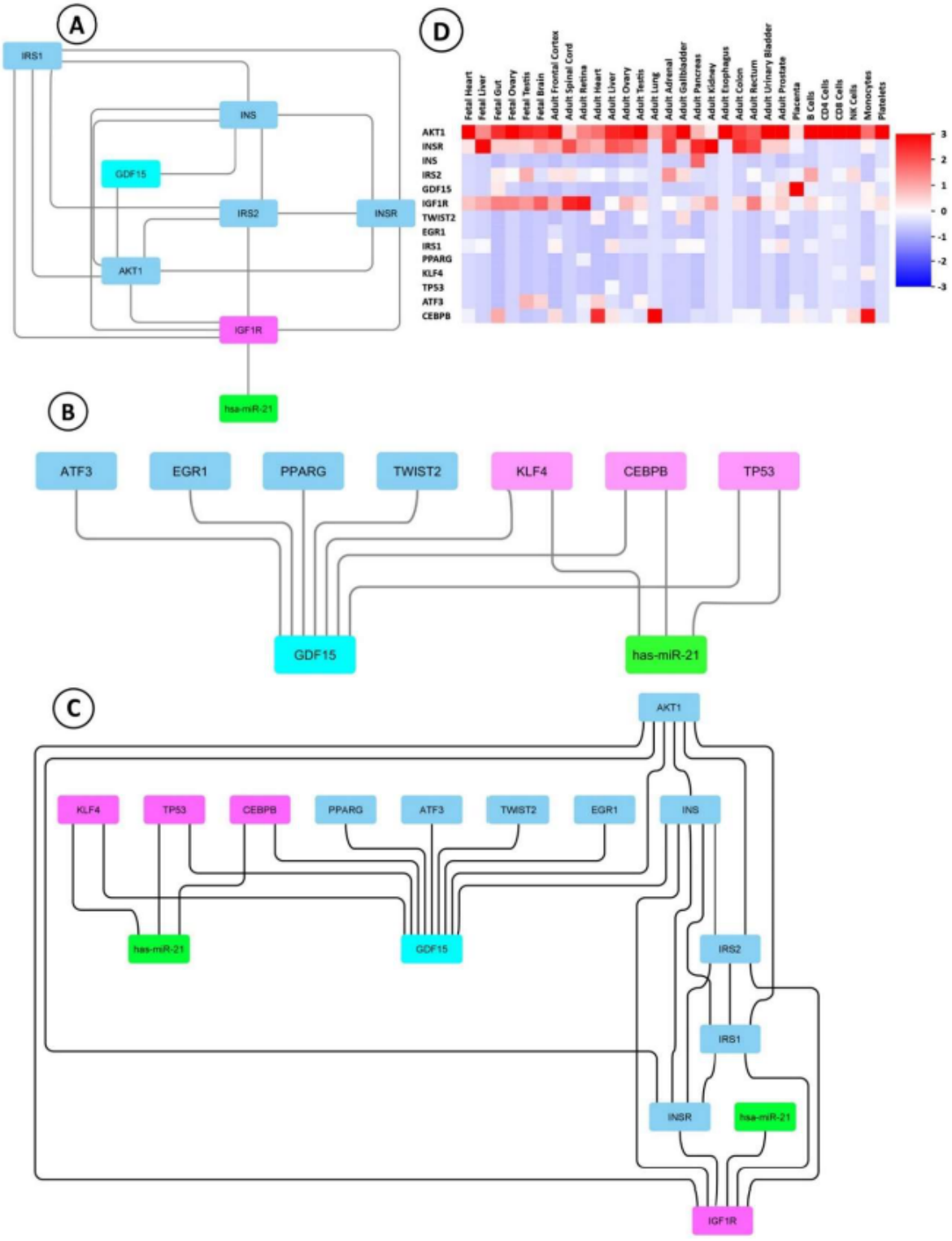

Figure 3

Protein-Protein interaction network of different genes (A) String PPIs network of Insulin, GDF-15 and networking genes with miRNA-21; (B) PPIs network of GDF-15 and Its transcription factor with common targeting miRNA-21; (C) Merged PPIs network of participating genes, GDF15 regulating transcription factor along with common targeting miRNA-21; (D) Heat map of different expression genes (DEGs) as obtained from PPI networks, in different organ of whole body in normal condition.

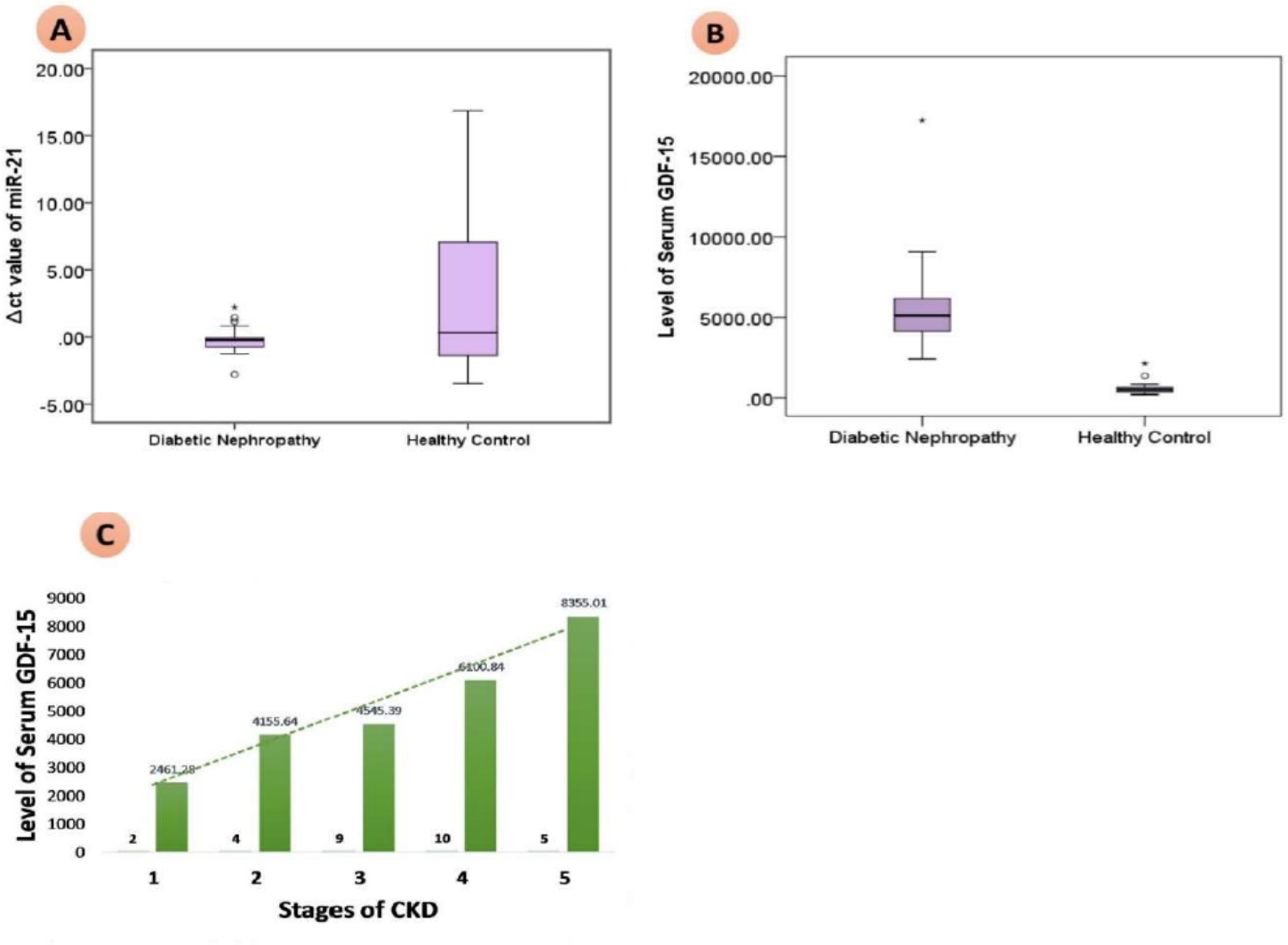

Figure 4

(A) Box -whisker plot of Δct value of miR-21 in DN patients and Healthy controls. The mean values of Δct miR-21 in DN (-0.26) and healthy controls (2.38) reveals an early expression of miR-21 in DN patients. (B) Box -whisker plot of serum levels of GDF-15 in DN patients (5507 ± 2759.89) and Healthy controls (567.36 ± 377.91); (C) Bar graph representing serum levels of GDF-15 in DN patients belonging to different stages of DN.

Supplementary Files

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- GraphicalAbstract.png