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Evaluating serum suPAR and urine miRNA-193a as a biomarkers for diagnosis of primary Focal Segmental glomerulosclerosis

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By

ALI RIDHA ABDULAMEER SHERALI

B.Sc. Medical Laboratory technology / 2013

Supervised by
Asst.prof. Dr.Ibrahim Abdul-Majeed Altamemi

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We, the members of examining committee, certify after reading this thesis (**Evaluating serum suPAR and urine miRNA-193a as a biomarkers for diagnosis of primary Focal Segmental glomerulosclerosis)** and after examining the student in its contents, it was found adequate for degree Master of Medical Microbiology with an excellent degree.

Signature
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Dr. Manal Mohammed kazem
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Chairman

Signature
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Dr. Mayada Farhan Darwish
Faculty of Science / University of Al-Kufa
Member

Signature
Assistant Professor
Dr. Mudhfer Mohammed Mazal
Faculty of Dentistry/university of Al-Qadisiyah
Member

Signature
Assistant Professor
Dr.Ibrahim Abdul-Majeed Altamemi
College of Medicine / University of Al-Qadisiyah
Member/Supervisor

Approval for the College Committee of Graduate Studies

Signature
Professor
Dr. Aqeel Reheem AL-Barqawi
Dean of the College of Medicine
University of Al-Qadisiyah

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا ۖ إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ (٣٢)

صَدَقَ اللهُ العَلِيُّ العَظِيم

(البقرة الآية ٣٢)

Supervisor's certification

I certify that, this thesis entitled " **Evaluating serum suPAR and urine miRNA-193a as a biomarkers for diagnosis of primary Focal Segmental glomerulosclerosis**" was prepared under my supervision at the Department of Medical Microbiology, College of Medicine /University of AL-Qadisiya , as partial fulfillment of the requirement for degree of Master of Medical Science in Medical Microbiology.

Signature

Assistant professor.

Dr. Ibrahim A. Mustafa
Department of Medical Microbiology,
College of Medicine
University of AL-Qadisiya
Date : / / /2018

Report of the Head of Department of Medical Microbiology

In view of the available recommendations, I forward this thesis for debate

by the examining committee.

Signature

professor

Dr .Adnan H. AL-Hamdani
Head of the Department of Medical Microbiology
College of Medicine
AL-Qadisiya University
Date : / / /2018

Dedication

To my father and my mother with all my love,

To my wife and children (ayah & naba)

To my brothers and all relatives,

To all my friends around the world,

To all of them, I dedicate my work

Ali sherali

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Summary

Summary

Focal segmental glomerulosclerosis is a progressive form of glomerular kidney disease resulting from hardening / scarring of glomeruli.

The renal biopsy is the definitive tool to diagnosis of FSGS that considered as invasive technique and requires surgical intervention.

The aims of present study are to investigate whether patients with focal segmental glomerulosclerosis (FSGS) have distinct circulating suPAR and urinary miRNA-193a expression profiles that could lead to a potential development of noninvasive diagnostic biomarkers of the disease. And finally to determine which of them have higher sensitivity and specificity by using healthy volunteers as a control group. To achieve this goal a blood samples as well as urine were collected from 24 Iraqi patient which include (13 male and 11 female) with primary FSGS ,who attended the consultant clinic of nephrology in AL-Diwaniyah teaching hospital in the period between 1January 2018 to 10 May 2018 under the supervision of nephrologist specialists were included in this study patients were diagnosed with FSGS according to histopathological report of kidney (biopsy) in addition to that the information about each case collected from patient .

In addition to that about 24 healthy volunteers were included as a control group. Blood samples were collected to be used for Enzyme linked immune sorbent assay test to determine concentration of soluble urokinase plasminogen activator receptor in serum while the urine samples were collected from (mid-stream) to be used for ribonucleic acid extraction quantitive polymerase chain reaction to study miRNA 193a in patient with primary FSGS.Data were summarized , presented and analyzed by using statistical package for social science (SPSS version 23)

The current study shows that the mean serum albumin was significantly lower in patients group than that in control group, 3.97 ±0.59

g/dl versus 4.68 ±0.89 g./dl (P = 0.002),in addition , the mean serum creatinine and mean blood urea were significantly greater in patients' group than in control group, 1.07 ±0.54 mg/dl versus 0.65 ±0.20 mg/dl and 37.79 ±8.33 mg/dl versus 27.25 ±70.95 mg/dl, respectively (P <0.05) and clinically detectable protein in urine (proteinuria) was limited to patients with glomerulonephritis. The level of miR193a in patients and control groups was 2.125 (5.86) fold change versus 0.375 (1.1) fold change, respectively and the difference was statistically highly significant (P < 0.001), being higher in patients' group than in control group, Moreover, the level of suPAR was also higher in patients than in control group, 7873.9 (2201) versus 3671.3 (1185.62), respectively; the difference was highly significant (P < 0.001)

To study the potential role of both miRNA 193a and suPAR in diagnosis and follow up of patients with glomerulonephritis, receiver operator characteristic revealed the cutoff value of miRNA193a was > 0.31 fold change with 100% sensitivity and 50% specificity; the AUC being 0.826 (95% confidence interval: 0.690-0.920) and hence an accuracy of 82.6 % and the significance level of (P <0.001). On the other hand, the cutoff value of suPAR was > 4610.15 with 100% sensitivity and 95.83 % specificity; the AUC being 0.998 (95% confidence interval: 0.923-1.000) and hence an accuracy of 99.8 % and the significance level of (P <0.001).

In conclusion, the suPAR is significantly more accurate, and also more specific for diagnosing patient with FSGS instead of the biopsy.

List of contents

list of table

List of Figure

List of Abbreviation

Abbreviation	Meaning
ACR	American college of Rheumatology
ACTN4	Actinin Alpha 4
APOL-1 gene	Apolipoprotein L1 gene
CD2	cluster of differentiation 2
CD2AP	CD2 associated protein
CLC-1	Cardiotrophin –like cytokine-1
ESRD	End stage of renal disorder
FSGS	Focal segmental glomerulosclerosis
GFR	Glomerular filtration rate
GPI	Glycosyl phosphatidyl inositol
MCKD	Minimal change kidney disease
NPHS1	Nephrin
ORG	Obesity – related glomerulopathy
PLCE1	Phosphatidylinositol-4,5-bisphosphate phosphodiesterase epsilon-1
PODXL	Podocalyxin-like protein 1

List of Abbreviation

Abbreviation	Meaning
RFT	renal function test
SCARB2	scavenger receptor class B member 2
suPAR	Soluble urokinase plasminogen activator receptor
TGF-β	Transforming growth factor beta
TRPC6	Transient receptor potential cation channel
UPA	Urokinase plasminogen activator
UTR	Untranslated region
WT-1	Wilms tumor

Chapter One

Introduction and literature review

Introduction:

The term focal segmental glomerulosclerosis (FSGS) is used to describe both a disease characterized by primary Podocytes injury and a lesion that occur secondarily to any type of chronic kidney disease(CKD), The most common clinical presenting feature of FSGS (>70% of patients) is nephrotic syndrome, characterized by generalized edema, massive proteinuria, hypoalbuminemia, and hyperlipidemia (Rosenberg et al. 2017).

Generally, FSGS is a progressive form of kidney disease, accounting for 2.3% of end-stage renal disease. (ESRD) The mechanisms of disease incompletely resolved but other evidence suggest that it involves immune cell dysfunction ,secretion of circulating factor and other factor (Kriz 2003).

The main problem in diagnosing FSGS is the use of harmful methods and invasive technique with requires surgical procedure to obtained on the sample thus now a day there is a new trends to find other non-invasive diagnostic methods for such disease .

In a study done by (Reiser et al) found that soluble urokinase plasminogen activator receptor(suPAR) identified as a circulating factor causing primary and recurrent FSGS. Circulating suPAR is elevated in approximately two thirds of primary FSGS, and it confers risk to both primary and recurrent FSGS. Further study is warranted to assess the role of soluble urokinase plasminogen activator receptor (suPAR) and its various pathological domains in FSGS risk stratification as well as in disease progression (Reiser et al. 2012).

Moreover, a study done by (Huang et al.) observed that suPAR a possible permeability factor ,in the plasma of patient with FSGS and determined their association with clinical and pathological date in 74 patients with primary focal segmental glomerulosclerosis (Huang et al. 2013).

Where, in a study done by (Wei et al.) have shown that suPAR is elevated in two- thirds of subjects with primary FSGS , but not in people with other glomerular

diseases, and have find that a higher concentration of soluble urokinase plasminogen activator receptor (suPAR) before transplantation underlies an increased risk for recurrence of focal segmental glomerulosclerosis (Wei et al. 2011).

In a study done by(Allison et al) to investigate the miR-193a induces a molecular cascade that leads to glomerular damage and focal segmental glomerulosclerosis (FSGS) in mice and human (Allison 2013) .

Moreover, a study done by (Huang et al.) they observed that glomerular up regulation of miRNA-193a has been detected in primary focal segmental glomerulosclerosis(FSGS) but not in other glomerular diseases and a significant increase in the levels of urinary exosomal miR-193a in primary focal segmental glomerulosclerosis (FSGS) patients compared to those in minimal change disease once was observed (Huang et al. 2017).

Thus, according to such contraversary the aims of present study are : Investigate whether patients with focal segmental glomerulosclerosis (FSGS) have distinct circulating suPAR and urinary miRNA-193a expression profiles that could lead to potential development of noninvasive diagnostic biomarkers of the disease. And finally determine which of them have higher sensitivity and specificity.

Objective of study:

1.Study the concentration of suPAR in patients serum by using ELISA test, and miRNA-193a in urine sample of patients with FSGS. And compared with control group.

2. using ROC analysis to find out the diagnostic sensitivity and specificity of both of these biomarkers

Focal segmental glomerulosclerosis

1.2 -Definition

Focal segmental glomerulosclerosis: it is major cause of progressive renal disease and end stage renal disorder with progressive glomerular scarring and proteinuria. (D'Agati et al. 2011) characterized by increased level of protein in urine (proteinuria) and defect in podocytes injury.(Fogo 2014)

other studies have shown that podocytes are main cell that cause development of Focal segmental glomerulosclerosis (Wharram et al. 2005). The patient with FSGS suffering from proteinuria ,hypoalbuminemia ,hypercholesterolemia and peripheral edema (Peired et al. 2013).

1.3-Classification of Focal segmental glomerulosclerosis :

Classification of FSGS is various and includes pathophysiologic, histologic, and genetic considerations (D'Agati et al. 2004). initially proposed that FSGS can be classified into primary (idiopathic) and secondary forms. The latter might be considered to involve familial/genetic forms, virus-associated forms, drug-induced forms, and forms mediated by adaptive structural-functional responses. clinical response may relate to the histologic variant, most notable the glucocorticoid responsiveness of the tip lesion and the aggressive of the collapsing variants ,more recently, pains to identify gentic diversity of FSGS in at risk population have acquired momentum with the most recent addition involving the APOL1 genetic variant as a major causes of FSGS in individual of sub-sahran African descent with FSGS. when putting together the genetic susceptibility ,pathophysiologic . clinical history and effect of the therapy, we believe that it is useful to the group , FSGS can be classified into six clinical forms including two groups the first common forms (primary FSGS and adaptive FSGS) and three less common forms (genetic FSGS, viral mediated FSGS ,and medication associated FSGS) (D'Agati et al. 2011).

FSGS can histologically be subdivided according to the Columbia classification into the following:

(i) Classical FSGS or FSGS NOS (not otherwise specified).

(ii) Collapsing variant (although there is discussion whether this is truly FSGS\ or rather a distinct pathology).

(iii) Tip variant.

(iv) Perihilar variant.

(v) Cellular variant (D'Agati et al. 2011).

other evidence including APOL1 associated FSGS with associated to primary FSGS some would prefer the term idiopathic FSGS both are define as a disease that arises spontaneously or is of unknown causes (McGrogan et al. 2011).

1.4-Epidemiology of disease :

In 1957, the ARNOLD RICH was first described the focal segmental glomerulosclerosis ,he hypothesized that the development of glomerulosclerosis accounted for the progression to end stage renal disorder seen in a group of children with idiopathic nephrotic syndrome . however it was not until the 1970s that FSGS appear as separate clinicopathologic entity according a report by the International study of kidney disease in children (Churg et al. 1970).

The regional incidence of ESRD in Northern Iraq is much lower than the crude incidences of 100 and 390 per million for Jordan and the US respectively. This is associated with low renal disease rates in the Iraqi elderly and an apparent major contribution of FSGS to ESRD (Ali et al. 2018).

The prevalence of FSGS in both adult and children in number of countries showing increase in worldwide and it is major causes leading to ESRD. However the incidence and prevalence of FSGS are difficult to achieve given the large global variation in the indication ,accessibility and pathology support for kidney biopsy (McGrogan et al. 2011) (Haas et al. 1997).

In some countries ,such as Brazil ,FSGS presently the most widespread as primary renal disease. (Bahiense et al. 2004) an analysis of the prevalence of glomerular disease in the united states caused by FSGS during 21 years period explain an increase from 0.2% in 1980 to 2.3% in 2000, and FSGS is most common primary glomerular disease leading to ESRD and proteinuria.

Although some change in prevalence of FSGS in countries may be related to changes in the biopsy exercise or disease classification .the primary FSGS is relatively more common in males than in female ,the incidence of glomerular disorder due to FSGS in males is 1.5 to 2 times higher than females , the incidence in both children and adult is more higher in blacks than in Caucasians .the factor responsible for increasing prevalence and incidence of FSGS are largely unknown (Rosenberg et.al 2017) .

another study demonstrates an increasing trend in FSGS incidence in Iranian children. However, kidney survival rates of our patients were similar to those reported by others in different countries The incidence rate of FSGS was 10.1% between 1982 and 1990, which was significantly increased to as high as 20.5% after the year 2000 (Hoseini et al. 2012).

1.5-Clinical manifestation of diseases :

FSGS is a defined by present the proteinuria typically accompanied by hypoalbuminemia , hypercholesterolemia and peripheral edema .in children 10% - 30% of patients with proteinuria are detected on routine checkups and physical examination.

In adult may detection in military induction examination , obstetric checkups and physical examination .The incidence of nephrotic- range proteinuria at onset in children is 70% to 90% whereas only 50% to 70% of adult with FSGS present with nephrotic syndrome. but in secondary forms of FSGS associated with hyperfiltration such as remnant kidney and ORG, typically to present lower levels of proteinuria and many such patient with FSGS have sub nephrotic proteinuria and a normal serum albumin concentration (Kambham et al. 2001) (Kopp et al. 2008).

The patient with FSGS suffering from present hypertension in 30% to 50% of children and adult, micro hematuria is present in 25% to 75% of these patients and the decrease of GFR is noted at presentation in 20% to 30% (Chun et al. 2004) (Meyrier 2005).

Daily urinary protein excretion ranges from less than 1 to more than 30 g/day .proteinuria is typically non selective and the complement levels and other serologic test results are normal ,but occasional patients will have glycosuria, aminoaciduria , phosphaturia or a concentrating defect explain functional tubular damage as well as glomerular injury (Valeri et al. 1996)(Schwimmer et al. 2003).

1.6-Etiology &risk factor

FSGS is a multi-factorial disease of different clinicopathologic syndrome with special etiologies that mentioned in the table (1.1).

Table(1-1) explain of etiologic classification of FSGS.(Fogo.2015)

Etiologic Classification of Focal Segmental Glomerulosclerosis	
Primary (Idiopathic) FSGS	Pamidronate
Probably mediated by circulating/permeability factor(s)	Sirolimus
Secondary FSGS	Anabolic steroids
1. Familial/Genetic*	***4. Mediated by Adaptive Structural-Functional Responses***
Mutations in nephrin *(NPHS1)*	*Reduced renal mass*
Mutations in podocin *(NPHS2)*	Oligomeganephronia
Mutations in α-actinin 4 *(ACTN4)*	Very low birth weight
Mutations in transient receptor potential cation channel *(TRPC6)*	Unilateral renal agenesis
Mutations in Wilms tumor suppressor *(WT1)*	Renal dysplasia
Mutations in inverted formin-2 *(INF2)*	Reflux nephropathy
Mutations in phospholipase C epsilon 1 *(PLCE1)*	Sequela to cortical necrosis
Risk alleles for apolipoprotein L1 *(APOL1)*	Surgical renal ablation
2. Virus Associated	Chronic allograft nephropathy
HIV-1 ("HIV-associated nephropathy")	Any advanced renal disease with reduction in functioning nephrons
Parvovirus B19	*Initially normal renal mass*
Simian virus 40 (SV40)	Hypertension
Cytomegalovirus (CMV)	Atheroemboli or other acute vaso-occlusive processes
3. Drug Induced	Obesity
Heroin ("heroin-nephropathy")	Increased lean body mass
Interferon	Cyanotic congenital heart disease
Lithium	Sickle cell anemia

1.6.1-genetic susceptibility or causes :

Mutation in different type of Podocyte genes have been specified in FSGS table (1.2) in children with nephrotic syndrome are often screened for mutations in the *NPHS1*genes that effect on nephrin and /or podocine because of the high

frequency of these mutation with high risk to development of FSGS recurrence(Kaplan et al. 2000) .

Other gene that cause FSGS is *CD2AP* gene that encode CD2-associated protein ,which links the slit diaphragm protein to the cytoskeleton have been detected in adult patients with proteinuria.(Kim et al. 2003)(Gigante et al. 2009)

Since the discovery of nephrin as the most important element of the slit diaphragm in 1998 (Kestila et al. 1998). The number of identified Podocytes mutation in familial and sporadic FSGS grown and the gene encode different Podocytes products located in the slit diaphragm, cell membrane , cytosol , actin cytoskeleton , nucleus , mitochondria and lysosomes. (Hinke et al. 2006)(Berkovic et al. 2008)(Santin et al. 2009).

Also a study done by (Santin et al) confirmed that mutation in nephrin and podocin are the most frequent to develop of FSGS (Santin et al. 2011).

Most mutations follow an autosomal recessive transmission and manifest early in the life .mutation in actinine -4 produce a rigid cytoskeleton by detected a buried actin- binding site that is in dependent of calcium regulation leading to gain of function (Weins et al. 2007).

table (1.2) explain genetic susceptibility of FSGS. (Kaplan et al. 2000)

Gene	Protein	Function	Phenotype
NPHS1	Nephrin	Podocyte slit diaphragm	Congenital nephrotic syndrome Finnish type, sporadic FSGS or nephrotic syndrome
CD2AP	CD2-associated protein	Podocyte slit diaphragm	Autosomal-dominant or autosomal-recessive sporadic adult-onset FSGS
NPHS2	Podocin	Podocyte slit diaphragm	Early onset autosomal-recessive FSGS
ACTN4	α-actinin-4	Podocyte cytoskeleton	Adult onset autosomal-dominant FSGS
MYO1E	Unconventional myosin 1E	Actin function	Early onset autosomal-recessive FSGS
INF2	Inverted formin-2	Actin regulation	Adult onset FSGS
PTPRO	Receptor-type tyrosine-protein phosphatase O*	Podocyte signalling	Autosomal-recessive childhood FSGS
ARHGDIA	Rho GDP-dissociation inhibitor 1	Rho GTPase signalling, actin dynamics	Early onset nephrotic syndrome or FSGS
TRPC6	Transient receptor potential channel 6	Calcium channel, podocyte mechanosensing	Autosomal-dominant or autosomal-recessive sporadic adult onset FSGS
WT1	Wilms tumour protein	Podocyte development	Autosomal-dominant sporadic FSGS, diffuse mesangial sclerosis
PLCE1	Phospholipase Cε1	Podocyte differentiation, signalling	Early onset autosomal-recessive FSGS or diffuse mesangial sclerosis
LMX1B	LIM homeobox transcription factor 1-β	Podocyte and GBM development	Nail–patella syndrome, rare FSGS
CD151	CD151 antigen	Podocyte and GBM, laminin–integrin interactions	Early FSGS, deafness, β-thalassemia
LAMB2	Laminin B2 chain	Interacts with integrin α3β1, links GBM to actin cytoskeleton	Autosomal-recessive Pierson syndrome or FSGS
ITGB4	Integrin β4	Cell–matrix adhesion	Rare FSGS
SMARCAL1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1	Chromatin bundling and gene transcription	Autosomal-recessive Schimke immunoosseous dysplasia, childhood FSGS
COQ2	Polyprenyltransferase	Mitochondrial function, deficient Coenzyme Q10	Autosomal-recessive early onset nephrotic syndrome or FSGS
COQ6	Ubiquinone biosynthesis monooxygenase COQ6	Ubiquinone biosynthesis	Autosomal-recessive nephrotic syndrome, FSGS, deafness
PDSS2	Decaprenyl diphosphate synthase subunit 2	Coenzyme Q10 synthesis, mitochondrial function	FSGS or collapsing FSGS
ADCK4	AarF domain-containing protein kinase 4	Coenzyme Q10 modulation	FSGS
MTTL1	Mitochondrially encoded tRNA leucine 1	Mitochondrial tRNA	Autosomal recessive MELAS or FSGS
SCARB2	Scavenger receptor class B member 2	Putative lysosomal receptor	FSGS or collapsing FSGS
APOL1	Apolipoprotein L1	Function unknown	Risk of FSGS, collapsing FSGS or HIVAN

*Also known as glomerular epithelial protein 1. Abbreviations: FSGS, focal segmental glomerulosclerosis; GBM, glomerular basement membrane; HIVAN, HIV-associated nephropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes.

1.6.2- Loss of Filtration Barrier:

Nephrotic proteinuria results from loss of integrity of the glomerular filtration barrier, which regulates perm selectivity through the intimate association of three layers: fenestrated glomerular endothelial cells at the inner blood interface, the glomerular basement membrane in the center, and podocytes (also known as visceral epithelial cells) at the outer urinary interface. Podocytes are highly differentiated, polarized epithelial cells resembling neurons in their large cell body and elongated cellular extensions, stabilized by a central actin cytoskeleton core (Tryggvason et al. 2006).

The foot processes interdigitate along the outer aspect of the glomerular capillary wall, linked to their neighbors by slit diaphragms, which are modified adherent junctions aligned in a zipper like array (Tryggvason et al. 2006). Podocytes provides structural support to the glomerular capillaries and synthesize the proteins of the slit diaphragm and many extracellular matrix components of the glomerular basement membrane. These terminally differentiated cells cannot repair by means of cell division, making podocytes depletion through detachment, apoptosis, or necrosis a critical mediator of glomerulosclerosis(Wiggins 2007).

1.6.3- Podocytes Depletion in Experimental Toxin Models:

experimental models have addressed whether delivery of a lethal toxin specifically and exclusively to the podocyte is sufficient to cause focal segmental glomerulosclerosis. For example, the creation of a transgenic animal that expresses a toxin receptor under the control of a Podocyte -specific promoter permits the targeting of a toxin exclusively to podocytes (Matsusaka et al. 2005)(Wharram et al. 2005).

The exotoxin A kills podocytes by the inhibition of protein synthesis. The degree of podocyte depletion after toxin exposure correlates closely with the severity of disease in these models. Loss of more than 40% of podocytes leads to overt focal segmental glomerulosclerosis with high-grade proteinuria and renal insufficiency, indicating a disease threshold (Wharram, Goyal et al. 2005).

Podocytes are shed into the urine for months after a brief to In a chimeric model in which only a subset of Podocyte express toxin receptor, Podocytes injury and dedifferentiation are observed to spread to neighboring toxin-resistant Podocyte that escaped the initial insult (Matsusaka et al. 2011).

This chimeric model suggests that injury can propagate locally from Podocyte to Podocyte by a domino-like effect, which may explain the segmental nature of the lesions. Although the mediators are unknown, Podocyte injury hypothetically might decrease Podocyte survival factors that signal through nephrin and glutamate receptors or might increase noxious factors, such as shear stress, angiotensin II, or transforming growth factor β (TGFβ) (Matsusaka et al. 2011).

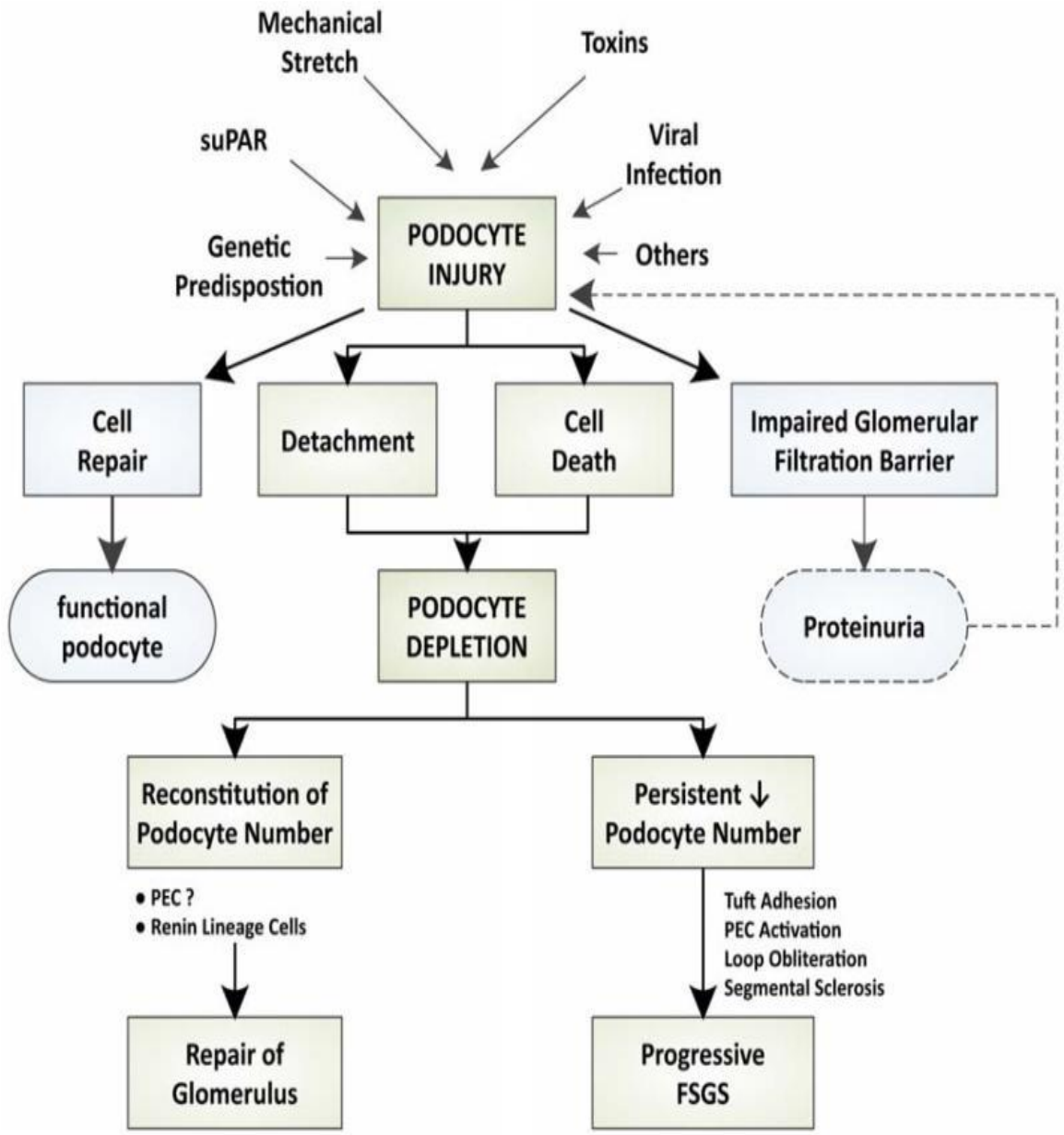
1.7-pathogenesis and causes of diseases:

The mechanisms of disease according to evidence suggest that it involves immune cell dysfunction ,secretion of circulating factor such as cardiotrophin-like cytokine-1 (CLC-1) , soluble urokinase plasminogen activator receptor (suPAR) ,vitronectin and integrin (Vinai et al. 2010) (Ponticelli 2010).

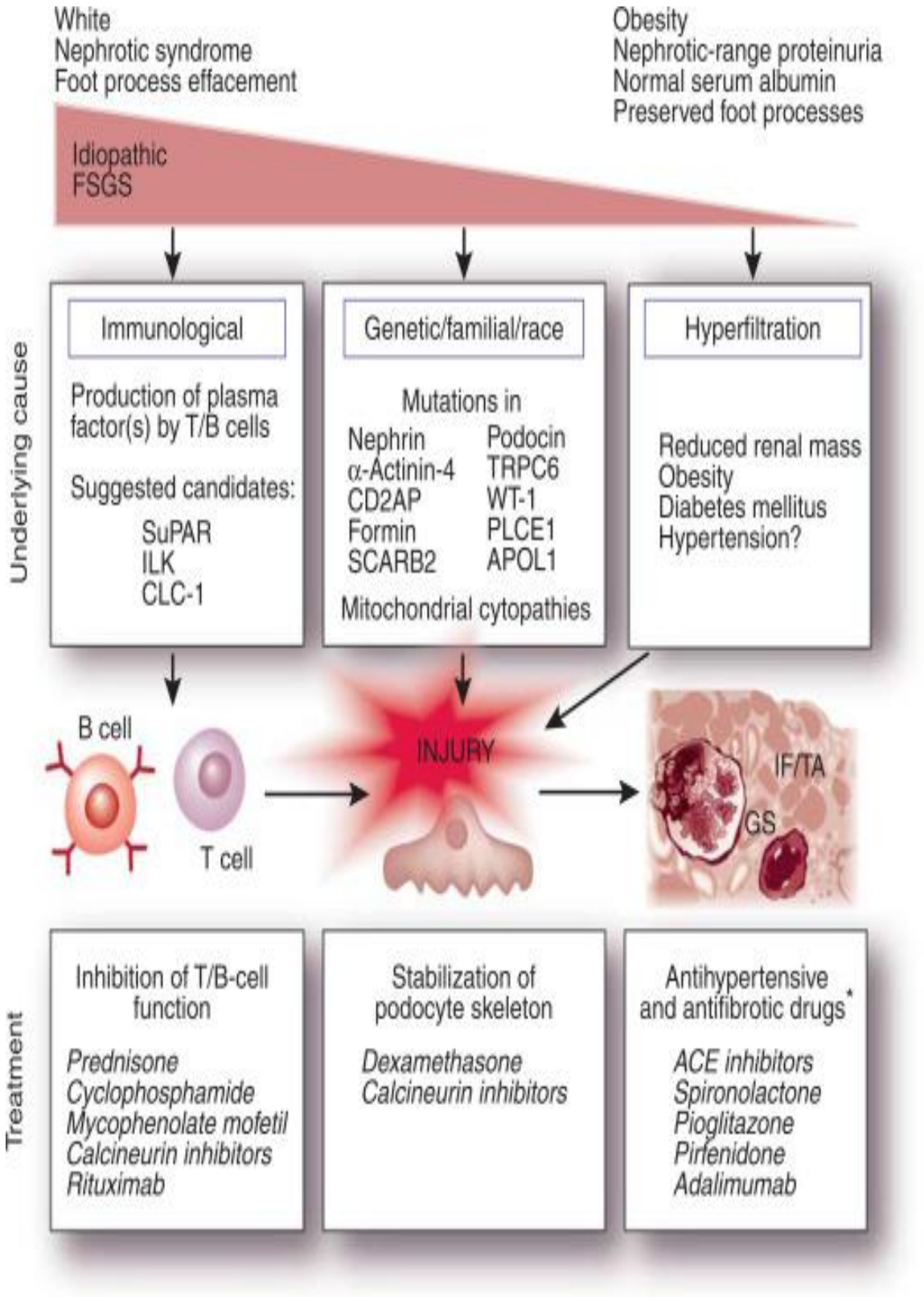
Other causes of disease include a genetic mutation in podocyte component , viral infection ,drug toxicities maladaptive to reduce number of functioning nephrons and hemodynamic stress placed on an at first normal nephron population .in all these form of FSGS may resulting from Injury or inherent within podocyte is a central pathogenic mediator(Meyrier 2005) (D'Agati et al. 2011) (Kim et al. 2016) .

These injuries elevate cell signaling re-organization of the actin cytoskeleton and producing foot process effacement .high levels of injury cause podocyte depletion through detachment or apoptosis. Stress placed on the remaining podocyte may spread to adjacent podocytes by reduction in important factor ,such as nephrin signaling or increase other factor such as toxic factors angiotensin II (Ang II) or mechanical strain on remnant podocyte (Matsusaka et al. 2011).

Cell-to-cell spread of podocyte injury untile all glomerular lobule is captured could demonstrate the characteristic segmental nature of the sclerosing lesion (D'Agati 2011).



Figure(1.1) explain the mechanisms of development of proteinuria in patient with FSGS. (D'Agati 2011)



Figure(1.2) explain the mechanisms the pathogenesis and treatment of FSGS. (Matsusaka et al. 2011).

1.8- diagnosis:

Biopsy is the golden diagnosis of patients with focal segmental glomerulosclerosis . After the appearance of clinical signs, for example, high protein content in the urine, high blood pressure (hypertension) and the appearance of blood in the urine(hematuria). And the study of chemical changes in the blood electrolytes that give indicators for people with this disease although biopsy is a harmful techniques that require surgical intervention and the insertion of a piece of kidney (Glomerular) and examined in the laboratory after long and complex procedures include fixation ,dehydration, clearing ,embedding, bloking , cutting, staining and examination under microscope by histopathologesit . Other modern methods include genetic screening of infected patients with FSGS by using modern techniques to identify genes responsible for genetic manipulation (Kim et al. 2016).

1-9 Criteria of good diagnostic marker.

Biomarkers introduction

In general, biomarkers are tools to detect whether a certain condition is present in the body. In a broad sense this can be about determining whether a certain process is taking place, , or levels of exposure to a substance. Biomarker literally means: ‘a biological compound that can be measured, which will reveal the presence and/or severity of a disease.

1-9.1 Biomarker purposes

Biomarkers can be used for different purposes. Based on the test objective, different biomarkers are appropriate. Biomarkers can be used to diagnose diseases, give a subtype classification, monitor diseases, predict the response to a certain treatment, and give a prognosis (LaBaer 2005).

1-9.2 Diagnosis of diseases

Biomarkers are used to diagnose diseases. In the clinic, biomarkers are used to distinguish between healthy and disease and to distinguish between diseases. This can lead to a problem for researchers trying to develop a new biomarker, as in the process of study the two groups that are contrasted are healthy people and people with the specific disease. This is a useful way to find new biomarkers and to study them. In the clinic the biomarker might not have the discriminating power that was observed under study circumstances. Biomarkers are needed for early detection of diseases. In the clinic, early diagnosis is relevant if early intervention is possible. FSGS diagnosis is based on biopsy and measurement of blood biomarker of renal disorder, among which renal tests are the most widely used. High –sensitivity renal tests have been developed , but they suffer from a lack of specifity since elevation of renal function test (RFT) can be due to non-related with FSGS. Therefore , there is an unmet need for novel early and specific biomarkers of FSGS .

It is important to develop early detection methods for etiopathogenesis of diseases such as FSGS .the ideal biomarker should be easily accessible, so that it can be sampled non-invasively and sensitive enough to detect the early presence of disease. Other studied this finding generated that circulating miRNA and suPAR may potential be used as non-invasive biomarker for diagnosis FSGS and other disease (Mitchell et al. 2008) .

1.10- soluble urokinase type plasminogen activator receptor:

suPAR is the soluble form of the urokinase type plasminogen activator receptor (uPAR) that may be glycosyl phosphatidylinositol (GPI) anchored three-domain membrane protein that can bind to many ligands including urokinase-type plasminogen activator , vitronectin or integrins (Thuno et al. 2009) .

uPAR is expressed not solely on varied immunologically active cells including monocytes, neutrophils, activated T lymphocytes and macrophages, however

additionally on endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, megakaryocytes, podocytes and bound tumor cells(Wei et al. 2008) (Thuno et al. 2009).

suPAR stems from split and release of the membrane-bound uPAR, and can be detected in plasma, urine, blood, serum, cerebrospinal fluid (C.S.F) and saliva. When the immune system is malfunctioning or activation, the levels suPAR concentration will increase, which has been documented in different pathological conditions paroxysmal nocturnal hemoglobinuria, HIV-1, certain bacterial infections, malaria and various types of cancers, and most recently also FSGS (Wittenhagen et al. 2004)(Sinha et al. 2014).

described the apperance of suPAR in plasma of healthy people and instructed that suPAR levels a reflection of overalluPAR-mediated cell surface chemical process(proteolysis). Soon, it became clear that suPAR will exist in several cleaved forms as well (Smith et.al 2010).

suPAR can be created by GPI-specific phospholipase-D-mediated cleavage of membrane uPAR in ovarian cells. Although the current concept is that suPAR is cleaved and released from cell-bound uPAR, also express suPAR that is being synthesized without the GPI anchor (Strausberg et al. 2002).

Whether this form also presenting in humans is unclear. uPAR is probably the most susceptible for cleavage at the linker region of DI connecting it with DII and DIII. The linker region can be proteolysis by several enzymes including uPAR, plasmin, chymotrypsin, different metalloproteinase and elastases (Behrendt et al. 1991).

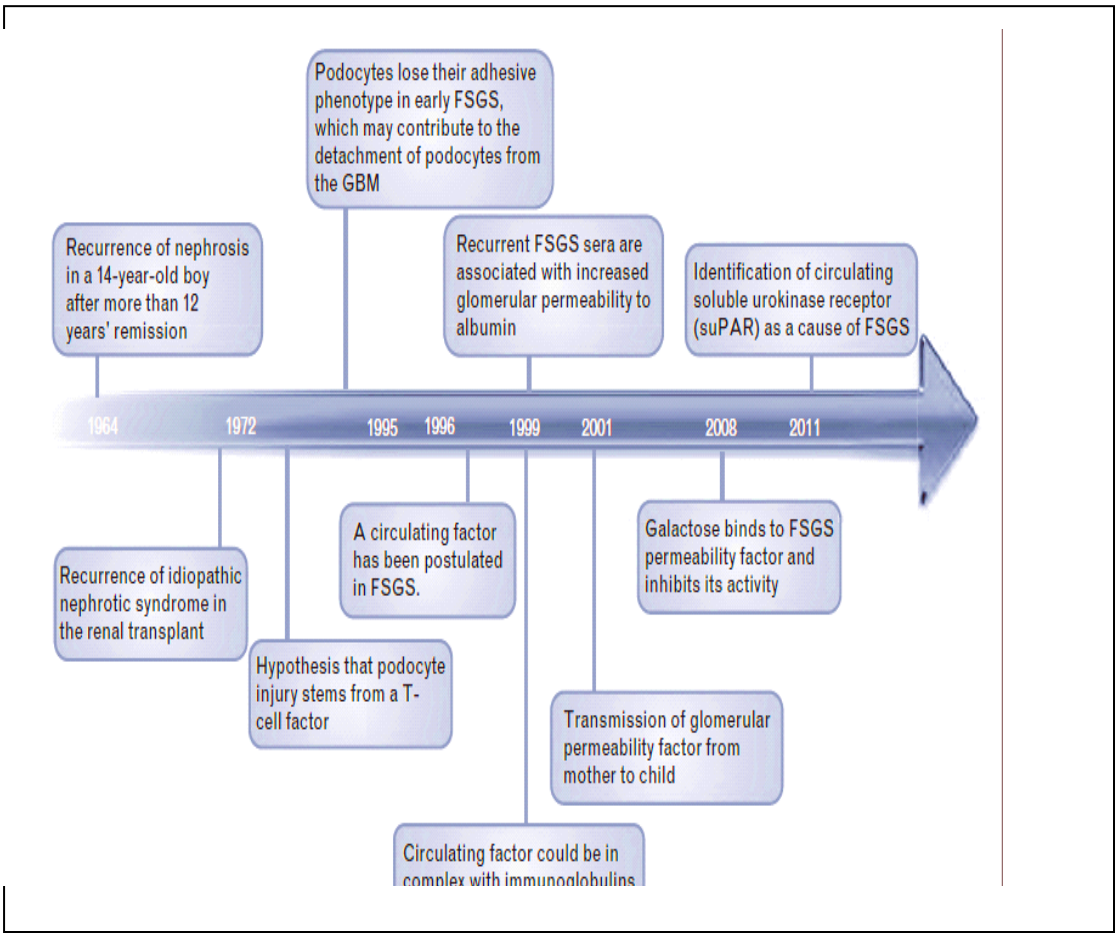


Figure (1.3) explain the concept that a circulating factor can cause pathologic permeability of the kidney . (Behrendt et al. 1991)

1-10-1 Role of suPAR in focal segmental glomerulosclerosis.

Both uPAR and suPAR exert various functions in basic cell motility. Cell migration is closely linked to adhesion and chemotaxis. uPAR is directly involved in these processes through both uPA-dependent and uPAR-independent interactions with integrins uPAR is distributed on the cell surface, and can be found in focal adhesions and at the leading edge of motile cells, wherein it can bind to integrins to regulate their activity state, which in turn determines ligand-binding affinity .(Rijneveld et al. 2002).

The reduced motility of podocytes in uPAR during injury can result in a stable glomerular filter, thus preventing proteinuria , Similarly, suPAR can bind to integrin, which in turn is responsible for integrin activation (Wei et al. 2008).

Pathological levels of serum suPAR mediate an elevated in activation of podocyte b3 integrin, shown to be a mechanism for podocyte foot process motility during the onset of native and post-transplant FSGS (Wei et al. 2011).

uPAR can be released from the plasma membrane by cleavage of the GPI anchor to act as a soluble molecule. suPAR can be further cleaved in the linker region between domains DI and DII, thereby releasing, for example, the fragments DI and DII DIII (Guo et al. 2015).

Moreover, an additional version, that is DI DII suPAR, can be found as a secreted version in rodents and this combination might also be present in other species. It is also possible that de-novo suPAR fragments can be produced during certain diseases including FSGS. Total serum suPAR levels were found to be significantly elevated in primary FSGS, but not in other glomerulopathies such as minimal change disease, membranous nephropathy and pre-eclampsia (Reiser et al. 2012) (Strausberg et al. 2002).

Furthermore, high pre-transplantation and post-transplantation serum suPAR levels were described in approximately 70% of FSGS patients and associated with heightened risk of FSGS recurrence, suggesting that suPAR contributes to both native and recurrent FSGS. The relevance of suPAR as a cause in primary FSGS was tested with the help of different experimental mouse models, showing that circulating suPAR could deposit in the glomeruli, binding to and activating podocyte avb3 integrin, causing foot process effacement and proteinuria.(Smith 2010). More importantly, overexpression of wild-type suPAR but not a b3 integrin binding-deficient point mutant in mice causes a glomerulopathy characteristic of FSGS (Wei et al. 2011).

The cellular source of elevated serum suPAR in FSGS is still unclear and needs further investigation, even though circulating monocytes and neutrophils are

likely implicated in the process. It could be that increased suPAR release is a result of some underlying infection in patients with FSGS, or of some persistent immune activation. integrin- mediated adhesion requires the activation of integrin heterodimers which involves conformational changes, thereby enhancing integrin affinity for ligands, a process termed integrin activation. The activation of avb3 integrin in podocytes is low under normal conditions and can be enhanced by ligands such as uPAR (in podocytes), or suPAR (from the circulation) Overly activated avb3 integrin causes small GTPase activation such as Rac-1 and podocyte foot process effacement and proteinuria (Peiredi et al. 2013).

1.10.2- clinical implications of suPAR:

In healthy individuals, suPAR levels are quite stable in both blood and urine. The measurement of suPAR can readily be done with a commercially available test . Like with any clinical test, one needs to determine first if the test result has implications in the management of the FSGS patient. Thus, it is important to realize that the three suPAR domains share a high degree of homology, and there is no specific antibody that selectively recognizes individual domains. Thus, the measurement of suPAR in most cases reflects the sum of all possible suPAR fragments. As more test systems might become available in the future, one needs to consider the test that best captures suPAR domains that are causing FSGS.

At present, we do not know which suPAR fragments exactly are present in FSGS patients and which of them are responsible for the Podocyte phenotype that is described. It appears that all suPAR domains in higher concentration cause proteinuria in mice, yet some of them with stronger effects than others . Thus, the higher the total suPAR concentration in the serum, the more likely also the negative effect of individual suPAR fragments on the glomerular filter. Given the relative importance of DII, a moiety that is required for binding to b3 integrin, it is reasonable to assume that DII-containing suPAR is critical for FSGS pathology. From a clinical point of view, it is of significant value to determine the circulating

suPAR level in the FSGS patient in an effort to perform risk stratification for the progression of the disease. However, there are no data yet indicating that higher suPAR levels lead to faster progression. Testing for suPAR might currently be best justified in the pretransplant evaluation wherein high levels might call for preemptive plasmapheresis, a treatment that can lower suPAR (Peiredi et al. 2013).

In this scenario, controlled prospective trials are warranted to evaluate if circulating suPAR testing and the necessitated suPAR lowering strategies will result in better outcomes or reduce the number of post-transplant FSGS recurrences. suPAR testing might also be of value in the management of post-transplant FSGS recurrence. Because suPAR can be partially removed by plasmapheresis, the measurements of suPAR before and after treatments are helpful to evaluate potential treatment efficacy. In this context, the application of patient sera before and after plasmapheresis can be applied on cultured human podocytes to study the degree of b3 integrin activation. The combination of both tests will allow to assess if plasmapheresis treatments are sufficient (when podocyte integrin activation is turned off) or not (when Podocyte integrin activation is still on). Another rationale for measuring suPAR in FSGS patients is the potential to use anti-suPAR strategies to treat or even cure FSGS. FSGS patients with elevated suPAR levels might eventually undergo treatment with the use of agents or mechanisms capable of blocking suPAR, its interaction with b3 integrin, reducing b3 integrin activity or removing suPAR from circulation. The latter might become critically important in the pre-transplant and post-transplant setting or to manage recurrent FSGS (Wei et al. 2011).

1.11-miRNA193a :

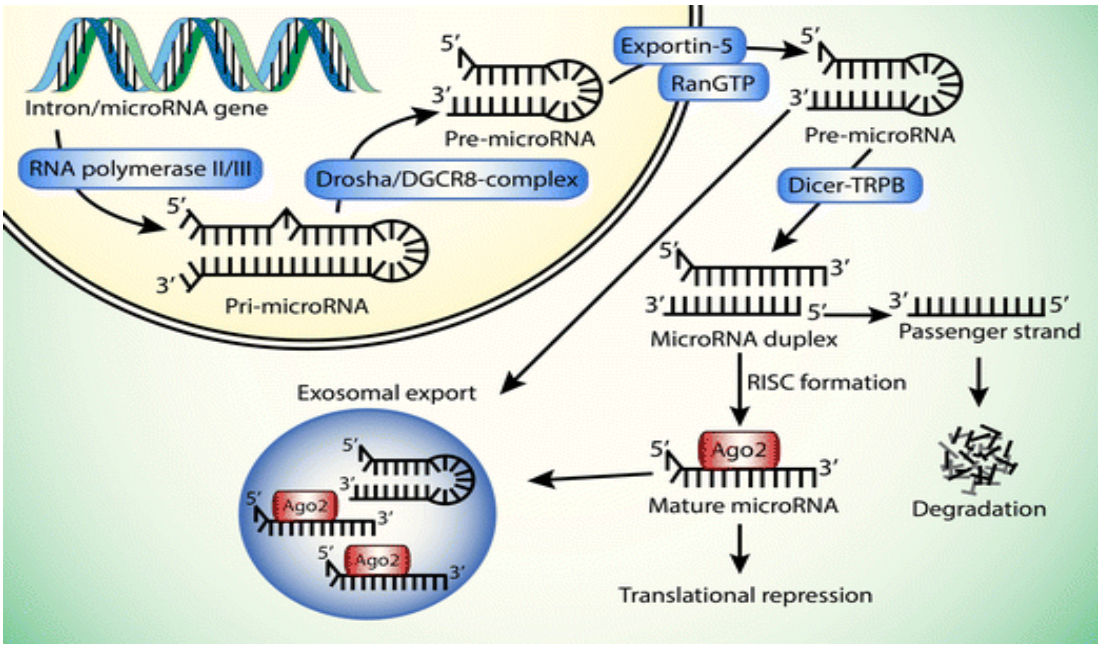
MicroRNAs comprise a large family of 21–22-nucleotide-long RNAs that have emerged as key post-transcriptional regulators of gene expression in animals and plants. In animals, microRNAs are predicted to control the activity of ~50% of

all protein-coding genes(Rajewsky 2006) . miRNA have many ideal characteristics as biomarker including their inherent stability and resilience(Wang et al. 2016). Inherent characteristics of microRNAs, such as their lower complexity, tissue-specific expression profiles, and stability, make these molecules ideal biomarkers to indicate various physiological and pathological states. The roles and functions of microRNAs may have broader implications; for example, microRNAs may serve as novel biomarkers for kidney disease prediction (Lee et al. 1993). Recent studies have suggested that microRNAs are potent regulators of the immune response, possessing great potential as both biomarkers and therapeutic targetsand are remarkably stable in the bloodstream. Circulating microRNAs have emerged recently as candidate biomarkers for disease, particularly cancer. In additional , the potential renal –related miRNA, were chosen according to previous miRNA (microarray) study (Kreth et al. 2018).

Functional studies indicate that microRNAs participate in the regulation of almost every cellular process, and are intrinsically associated with many human pathologies(Di Leva et al. 2014).

In animals, microRNAs are processed from longer hairpin transcripts, known as pre-microRNA, by the RNase III-like enzymes Drosha and Dicer, whereas in plants only Dicer is responsible for microRNA processing(Voinnet 2009, Krol, Loedige et al. 2010). One strand of the hairpin duplex is loaded into an Argonaute family protein (AGO) to form the core of microRNA-induced silencing complexes (MiRISCs). MiRISCs silence the expression of target genes through mRNA decay and translational repression. The target recognitions are achieved through base-pairing complementarity between the loaded microRNA and the target mRNA that contains a partially or fully complementary sequence**.** Unlike plant microRNAs, that recognize fully complementary binding sites within the open reading frame (ORF)**.** animal microRNAs recognize partially complementary binding sites generally located in the 3'-untranslated region (UTR) (Figure 1). For most microRNA binding sites, the complementarity is limited to the seed sequence found

in the 5'-end of microRNA from nucleotide 2 to 7. The partial recognition between microRNA and its target is sufficient to trigger silencing. (Ho et al. 2011)



Figure(1.4) MicroRNA target recognition mechanism: (Ho et al. 2011)

The significance of microRNA in renal pathophysiology has been demonstrated in Dicer knockout animal models. During kidney development, the global knockout of Dicer in nephron progenitor cells results in a marked decrease in nephron number.(Ho et al. 2011) Podocyte-specific loss of Dicer function causes proteinuria, foot process effacement, and glomerulosclerosis (Harvey et al. 2008)(Shi et al. 2008).

Deletion of Dicer in renin-secreting juxtaglomerular cells results in a selective loss of these juxtaglomerular cells, suggesting a role in cell fate determination. (Sequeira-Lopez et al. 2010) In the proximal tubule, microRNA appear to promote cellular injury because a selective loss of Dicer in animals after three weeks of age confers resistance to ischemia-reperfusion injury(Wei et al. 2010) .

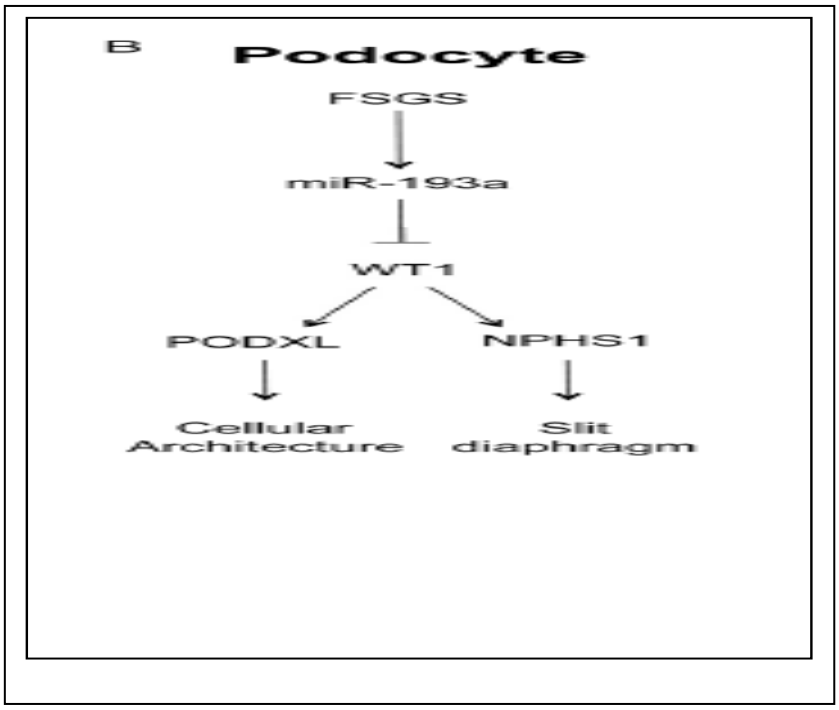


figure (1.5) focal segmental glomerulosclerosis (FSGS) induces the expression of miR-193a. (Sequeira-Lopez et al. 2010**)**

1.11.1-MicroRNA in Glomerular Podocyte:

Podocyte in the glomerular basement membrane are critical in the maintenance of structure and function of the glomerular filtration barrier. To study an overall role of miRNAs in podocyte biology, two independent lines of Dicer KO mice were generated for podocytes. Mutant mice developed proteinuria by three weeks after birth and progressed rapidly to end-stage kidney disease. Multiple abnormalities were observed in glomeruli of mutant mice, including foot process effacement, irregular and split areas of the glomerular basement membrane, podocyte apoptosis and depletion, mesangial expansion, capillary dilation, and glomerulosclerosis.(Shi et al. 2008) Focal segmental glomerulosclerosis (FSGS) is a devastating glomerular diseases caused by podocyte dysfunction. Deranged expression of several podocyte specific genes (WT1, NPHS1, ACTN4, and TRPC6), accompanied by collapse of normal Podocyte shape and Podocyte foot

process effacement, presents as major pathogenic origins for FSGS. The importance of microRNAs in FSGS has been demonstrated by (Gebeshuber and colleagues) in a recent study. Through transgenic screening in mice, (Gebeshuber *et al*.) have identified miR-193a as a powerful inducer of FSGS. Mechanistically, miR-193a silences the Wilms' tumor (WT1) gene, which encodes a transcriptional factor and acts as a master regulator for podocye homeostasis(Gebeshuber et al. 2013). In normal Podocyte, WT1 positively regulates the expression of several key genes crucial for Podocyte architecture, e.g. podocalyxin (PODXL) and for slit diaphragm formation, e.g., nephrin (NPHS1). The level of miR-193a was consistently higher in isolated glomeruli from FSGS patients compared to normal kidneys, which provides an important mechanism for FSGS pathogenesis (Figure 2B) (Gebeshuber et al. 2013) (Chen et al. 2011) .

Chapter Two
Materials and Methods

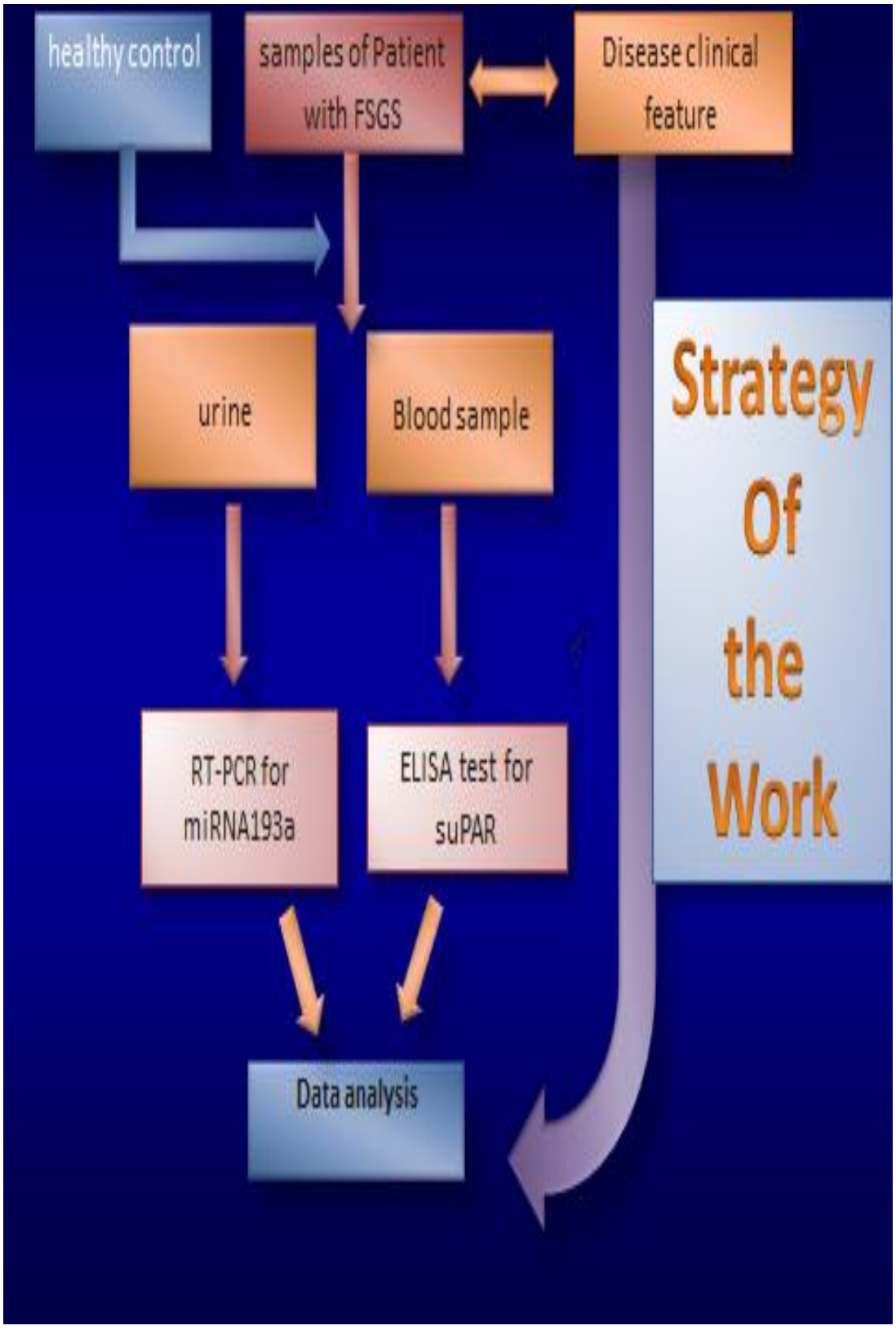
2- Materials and Method

2.1:patient and sample collection.

A case control study was conducted in AL-Diwaniyah province. A blood and urine samples from 24 Iraqi patients which include (13 male and 11 female) with primary FSGS, who attended the consultant clinic for nephrology in AL-Diwaniyah teaching hospital in the period between 1January 2018 to 10 May 2018 under the supervision of nephrologist specialists were included in this study patients were diagnosed with primary FSGS according to histopathological report of kidney (biopsy) under treatment in addition to that the information about each case collected from patient as well as the test such as urea, serum creatinine ,serum electrolyte , protein in urine and histopathological test performed in the hospital according to form that mentioned in appendix.

In addition to that about 24 healthy volunteers were included as a control group. Blood sample were collected by venipuncture from 24 patient and their healthy controls (five millimeter of venous were drawing by disposable syringe under aspect technique and putting in gel tube with allowed to clot then serum was separated by centrifugation 1500 rpm for 5 minute. The serum has been collected in eppendrof tube then stored at -20c to be used for ELISA test to determine concentration of suPAR in serum. While the urine sample were collected from (mid-stream) to avoid contamination take 5 ml in a sterile tube, must be centrifuge 1500 rpm for 5 minute and taken the supernants and discard the precipitate to be used for RNA extraction ,qPCR to study miRNA 193a in patient with primary FSGS under treatment . study design is shown in figure(2-1).

Figure (2-1): shows the study design



2.2. Material

Table (2-1): Instruments and equipment and remarked

No.	Instrument / equipment	Company / Country
1	Compound light microscope	Olympus (Japan)
2	Digital camera	Sony (Japan)
3	Oven	Memmert
4	High speed Cold Centrifuge	Eppendorf/ Germany
5	Incubator	Memmert (Germany)
6	Thermocycler apparatus	Bioneer/ Korea
7	Nano drop Spectrophotometer	Thermo Scientific/ UK
8	Vortex	CYAN/ Belgium
9	Micropipettes (different volumes)	Eppendorf / Germany
10	Eppendorf tubes	Sigma(England)
11	Hot plat stirrer	Labtech /Korea
12	Sensitive balance	Sartorius (Germany)
13	Miniopticon Real Time PCR	Bio-Rad/ USA
14	Water bath	Kottermann (Germany)
15	Exispin vortex centrifuge	Bioneer/ Korea
16	Refrigerator	Concord/ lebanon
17	Automatic ELISA Reader	Paramedical/ ITalian
18	Plain tube	AFCO-DISPO/Jorden

2.3. Kits

Table (2-2): The kits used in this study with their companies and countries of origin:

No.	Kit	Company	Country
1	Total RNA Extraction Kit AccuZolTM	Bioneer	Korea
	Trizol reagent 100ml		
2	DNase I enzyme kit	Promega	USA
	Dnase I enzyme		
	10x buffer		
	Free nuclease water		
	Stop reaction		
3	M-MLV Reverse Transcriptase kit	Bioneer	Korea
	M-MLV Reverse Transcriptase (10,000U)		
	5X M-MLV RTase reaction buffer		
	dNTP		
	100mM DTT		
	RNase Inhibitor		

NO	AccuPower ® Plus DualStarTM qPCR PreMix	Bioneer	Korea
	qPCR PreMix for TaqMan probe: Taq DNA polymerase dNTPs (dATP, dCTP, dGTP, dTTP) and 10X buffer		
	DEPC water		
	8-well strip, 50$\mu\ell$/rxn, optical film included		

2.4. Primers and probes

2.4.1. microRNA Primers and probes

The Primers and probes for microRNA were design in this study by using **(The Sanger Center miRNA database Registry**) to selected miRNA sequence **and using miRNA Primer Design Tool.** These primers and probe were provided by (Bioneer company, Korea) as following table:

Primer	**Sequence**	
hsa-miR-193a RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGC ACCAGAGCCAACTCAACA	
hsa-miR-193a primer	F	GTTTGGTAGCTTATCAGACTGA
	R	GTGCAGGGTCCGAGGT
hsa-miR-193a probe	FAM- CCAGAGCCAACTCAACA-MGB	

2.4.2. GAPDH gene Primers and probes

The GAPDH gene Primers and probes were designed by using NCBI- Gene Bank data base and Primer 3 design online**.** These primers were provided by (Bioneer company, Korea) as following table:

Gene	Sequence	
GAPDH primer	F	TCAGCCGCATCTTCTTTTGC
	R	TTAAAAGCAGCCCTGGTGAC
GAPDH probe	FAM- CCAGCCGAGCCACATCGCTC-TAMRA	

2.5. Chemicals

The chemical and biological materials used in this work are listed in Table(3-2) below:

Table (2-3): Chemical materials with their remarks

No.	Chemicals	Company	Country
1	Chloroform	Labort	India
2	DEPC water	Bioneer	Korea
3	Isopropanol	Labort	India
4	Ethanol 100%	Labort	India
5	RNase free water	Bioneer	Korea

2.6- Human suPAR ELISA Kit

Human suPAR ELISA kit was used in this study for quantitative determination of suPAR concentration in serum of FSGS and their control group and procedure has been done according to manufactural instruction as mentioned below:

2.6.A. suPAR Human ELISA Kit with its component:

Reagent	Quantity
Micro ELISA Plate	8 wells x12 strips
Reference Standard	2 vials
Reference Standard & Sample Diluent	1vial 20mL
Concentrated Biotinylated Detection Ab	lvial 120qL
Biotinylated Detection Ab Diluent	1vial 10mL
Concentrated HRP Conjugate	lvial 120qL
HRP Conjugate Diluent	1vial 10mL
Concentrated Wash Buffer (25 x)	1vial 30mL
Substrate Reagent	1vial 10mL
Stop Solution	1vial 10mL

2.6.B:principle of assay

This assay employs the quantitative sandwich enzyme immunoassay techniques. The micro ELISA plate has been pre-coated with an antibody specific to suPAR. Then antigen is bound to immobilized capture antibody, standard and samples are pipetted into the well and any suPAR present is bound by the immobilized antibody .After removing any unbound substance by simple washing procedure. Biotin -conjugated antibody specific for suPAR is added to the wells. After washing ,

Avidin conjugated Horse radish peroxidase (HRP)was add to each microplate well and then incubated and then wash to remove any unbound Avidin -enzyme reagent ,substrate solution was added specific to the enzyme in the well . The color intensity produced is directly proportional to the amount of suPAR bound in the initial step. The enzyme-substrate reaction is terminated by the addition of a stop solution and the color turns yellow.The optical density (OD) is measured spectrophotometrically at a wave length of 450nm .The OD value is proportional to the concentration of suPAR and then calculate the concentration of suPAR in the sample by comparing the OD of the samples to the standard curve.

2.6.c: procedure assay: before begging the assay, all kit reagents and samples were bring at room temperature.

1. **Sample:** 100pL of Standard, Blank, or Sample were added per micro ELISA plate well. The blank well was filled with Reference standard and Sample diluent. After that solutions mixed gently and cover the plate with sealer, and then incubated for 90 minutes at 37°C and then made wash to remove unbound reagent .

2. **Biotinylated Detection Ab:** The liquid of each well were removed, and immediately 100pL Biotinylated Detection Ab working solution was added to each well and covered with the plate sealer and then incubated for 1 hour at 37°C.

3.wash: All plate wells were aspirated and washed, and repeated the process three times. The wash done by filling each well with Wash Buffer (approximately 350pL) using a squirt bottle.

4. HRP Conjugate: 100pL of HRP Conjugate working solution was added to each well and covered with the plate sealer. And then incubated for 30 minutes at 37°C.

5.Wash: The wash process was repeated for five times as conducted in step3.

6. Substrate: 100pL of substrate Solution was added to each well and covered with a new Plate sealer, then incubated for about 15 minutes at 37°C.

7. **Stop**: 100pLof Stop Solution was added to each well. Then, the color turns to yellow immediately.

8. **OD Measurement**: for determing the optical density (OD value) of each well at once, used a micro-plate reader set at 450 nm.

2.6.d: **Calculation of results:**

The ELISA results were calculation depend on the average of the duplicate readings for each standard and samples optical density. Then create a standard curve by plotting the mean OD value for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph in excel office program.

1-First, calculated the mean O.D value for each standard and sample ,then constract the standard curve.

2-determined the amount of suPAR in each sample, by locating the O.D according to the equation Y=4029.3X for suPAR.

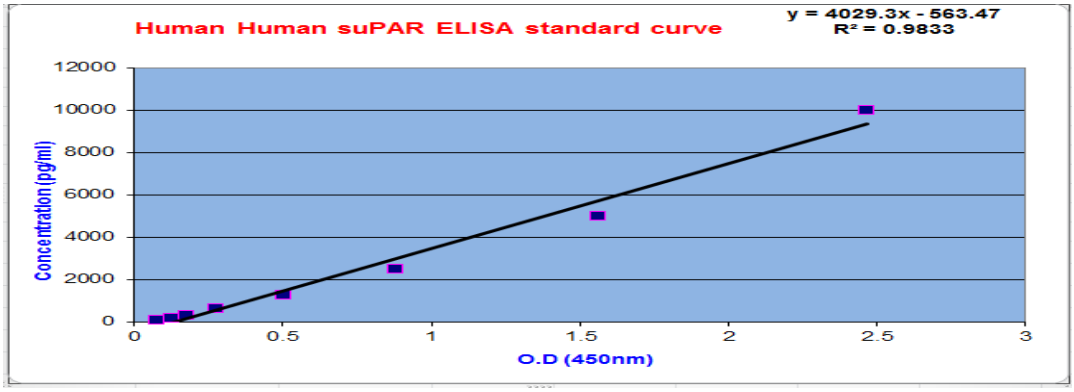


Figure 2-2: standard curve of suPAR..

2.7. Total RNA extraction

Total RNA were extracted from urine samples by using (TRIzol® reagent kit. Bioneer. Korea) and done according to company instructions as following steps:

1- 250µl urine samples was placed in 1.5 micro centrifuge tube then 750µl TRIzol® reagent was added to each tubes.

2- Then, 200µl chloroform was added to each tube and shaken vigorously for 60 seconds.

3- The mixture was incubated on ice for 5 minutes. Then centrifuged at 12000 rpm, 4C°, for 15 minutes.

5- Supernatant was transferred into a new eppendorf tube, and 500µl isopropanol was added. Then, mixture mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes. Then, centrifuged at 12,000 rpm , 4C° for 10 minutes.

8- Supernatant was discarded, and 1ml 80% Ethanol was added and mixed by vortex again. Then, centrifuge at 12000 rpm, 4C° for 5 minutes.

9- The supernatant was discarded and the RNA pellet was left to air to dry.

10- 50µl DEPC water was added to each sample to dissolve the RNA pellet, Then, the extracted RNA sample was kept at -20.

2.7.1. Estimation RNA yield and quality

The extracted genomic RNA was checked by using Nanodrop spectrophotometer (THERMO. USA) that check RNA concentration and estimation of RNA purity through reading the absorbance in at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application.

2. A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 2µl of ddH2O onto the surface of the lower measurement pedestal.

3. The sampling arm was lowered and clicking OK to blank the Nanodrop, then cleaning off the pedestals.

4. After that, the pedestals are cleaned and pipet 1µl of RNA sample for measurement.

2.7.2. DNase I Treatment

The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by Promega company, USA instructions as follow:

Mix	Volume
Total RNA 100ng/ul	10ul
DNase I enzyme	1ul
10X buffer	4ul
DEPC water	5ul
Total	20ul

After that, The mixture was incubated at 37C° for 30 minutes. Then, 1µl stop reaction was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

2.7.3. cDNA synthesis

2.7.3.1 cDNA synthesis for miRNA

DNase-I treated RNA samples were used in miRNA cDNA synthesis step for miRNA by using **M-MLV Reverse Transcriptase kit** and done according to company instructions as following tables:

Step 1

RT master mix	Volume
Total RNA 100ng/ul	8ul
hsa-miRNA 193a RT primer	1ul
DEPC water	1ul
Total	10ul

Step 2:

RT master mix	Volume
Step 1 RT master mix	10ul
M-MLV RTase (200u)	1ul
5X M-MLV RTase reaction buffer	4ul
100mM DTT	2ul
dNTP	2ul
RNase inhibitor	1ul
Total	20ul

Than RNA and primer was denatured for 10 min at 65 °C, after that immediately cool on ice.

Then the tubes were placed in vortex and briefly spinning down. The RNA converted into cDNA in thermo cycler under the following thermo cycler conditions:

Step	Temperature	Time
cDNA synthesis (RT step)	42 °C	1 hour
Heat inactivation	95 °C	5 minutes

2.7.3.2. cDNA synthesis for GAPDH gene

DNase-I treated RNA samples were also used in cDNA synthesis step for GAPDH gene by using **M-MLV Reverse Transcriptase kit** and done according to company instructions as following tables:

Step 1

RT master mix	Volume
Total RNA 100ng/ul	8ul
Random Hexamer primer	1ul
DEPC water	1ul
Total	10ul

Than RNA and primer was denatured for 10 min at 65 °C, after that immediately cool on ice.

Step 2

RT master mix	Volume
Step 1 RT master mix	10ul
M-MLV RTase (200u)	1ul
5X M-MLV RTase reaction buffer	4ul
100mM DTT	2ul
dNTP	2ul
RNase inhibitor	1ul
Total	20ul

Then the tubes were placed in vortex and briefly spinning down. The RNA converted into cDNA in thermocycler under the following thermocycler conditions:

Step	Temperature	Time
cDNA synthesis (RT step)	42 °C	1 hour
Heat inactivation	95 °C	5 minutes

2.8. STEM-LOOP RT-qPCR

The stem loop RT-qPCR was used in quantification of 193a miRNA expression analysis that normalized by housekeeping gene (GAPDH) in urine with primary FSGS patients and normal urine samples by using Real-Time PCR technique and this method was carried out according to

method described by (Gebeshuber et al.2013) and include the following steps:

1- qPCR master mix preparation

qPCR master mix was prepared by using **AccuPower ® Plus DualStar™ qPCR PreMix kit** that dependant on TaqMan probe FAM dye detection of gene amplification in Real-Time PCR system and include the follow:

qPCR master mix	Volume
193a miRNA cDNA template (100ng)	5µL
Forward primer(10pmol)	2.5 µL
Reverse primer (10pmol)	2.5 µL
TaqMan probe (20pmol)	2.5 µL
DEPC water	25 µL
Total	50 µL

After that, these qPCR master mix component that mentioned above placed in qPCR premix standard plate tubes that contain the other PCR TaqMan probe amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, than placed in Miniopticon Real-Time PCR system.

2- miRNA qPCR Thermocycler conditions

After that, the qPCR plate was loaded and the following thermocycler protocol in the following table:

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	45
Annealing\Extention Detection(scan)	55 °C	30 sec	

2.9. Quantitative Real-Time PCR (qPCR)

The quantitative Real-Time PCR used in quantification of housekeeping gene (GAPDH) that used in normalization of 193a miRNA expression analysis in urine primary FSGS patients and normal urine samples. this method was carried out according to method described by (Gebeshuber et al.2013) and include the following steps:

1- qPCR master mix preparation

qPCR master mix was prepared by using **AccuPower ® Plus DualStar™ qPCR PreMix kit** that dependant on TaqMan probe FAM dye detection of GAPDH gene amplification in Real-Time PCR system and include the follow:

qPCR master mix	volume
cDNA template (100ng)	5µL
Forward primer(10pmol)	2.5 µL
Reverse primer (10pmol)	2.5 µL
TaqMan probe (20pmol)	2.5 µL
DEPC water	25 µL
Total	50 µL

After that, these qPCR master mix component that mentioned above placed in qPCR premix standard plate tubes that contain the other PCR TaqMan probe amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, than placed in Miniopticon Real-Time PCR system.

2- **qPCR Thermocycler conditions**

After that, the qPCR plate was loaded and the following thermocycler protocol in the following table:

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	45
Annealing\Extention Detection(scan)	55 °C	30 sec	

2.10. Data analysis of qRT-PCR

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) by using The ΔCT Method Using a Reference that described by (Livak and Schmittgen, 2001). The relative quantification method, quantities obtained from q RT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels. After that, the ΔCT Method with a Reference Gene was used as following equations:

Gene	Test (Treatment group)	Cal. (Control group)
Target gene	CT (target, test)	CT (target, cal)
Reference gene	CT (ref, test)	CT (ref, cal)

Relative quantification (the ΔΔCT method)

The ΔΔCT method, also referred to as the Comparative CT method, is a means of measuring relative quantification and was described by Livak and Schmittgen 2001. It determines the relative change in gene expression between a target gene under investigation and that of calibrator (control) gene. Most frequently, the untreated control is used as the calibrator.

The difference between the CT of the target gene (CT, target) and the CT of the endogenous control (CT, ec) is the ΔCT of the sample:

ΔCT = CT, target - CT, ec

The term ΔΔCT is calculated as the ΔCT of the target gene in the treated sample minus the ΔCT of the target in the untreated, calibrator sample:

ΔCT = ΔCT, target in treated sample - ΔCT, target in calibrator sample

The calibrator, since it is untreated, should have no change in its ΔΔCT value during the course of the experiment. Its change, therefore, is equivalent to zero. Since 20 equal one, the calibrator gene's expression is unity.

When the ΔΔCT method is used to measure gene expression, therefore, the results are expressed as a " fold" change in the expression level of the target gene normalized to the endogenous control and relative to the calibrator. It is given by the equation:

Relative Fold Change =- 2 ΔΔCT

The amplification efficiency (E) for the sequence of interest was calculated by the PCR machine software

.

2.11- Statistics analysis :were summarized, presented and analyzed using statistical package for social science (SPSS version 23) and Microsoft Office Excel 2010. Numeric data were presented as mean, standard deviation, median and interquartile range (IQR) while nominal data were expressed as number and percentage . Independent sample T-test was used to compare mean value between two groups while Mann Whitney U test was used to compare median value between two groups. Correlation cofficiant was estimated by spearman correlation .

ROC analysis was done to determine cutoff value predict positive diagnosis . The level of significance was considered at P-value >0.05 was considered significant .

Chapter Three

Results and Discussion

3-Results and Discussion

3.1 demographic Characteristic of the studied samples.

Table 3.1 shows the summary of demographic information of patients with focal segmental glomerulosclerosis (FSGS) population and healthy control.

Table 3.1 Demographic Characterization of the studied samples.

Characteristic	Patients n = 24	Control n = 24	*P*
Age			
Mean ±SD years	28.38 ±13.70	30.38 ±12.78	0.604† NS
Range (min.-max.) years	(12 -55)	(14-55)	
Gender			
Male, n (%)	13 (54.2%)	19 (79.2%)	0.066¥ NS
Female, n (%)	11 (45.8%)	5 (20.8 %)	

n: number of cases; min.: minimum; max.: maximum; † Independent samples t-test; ¥ Chi-Square test; NS: not significant

In the above table, The age of patients ranged from 12 to 55 years and that of control subjects ranged from 14 to 55 years. There was no significant difference in mean age of patients and control groups including 28.38 ±13.70 years versus 30.38 ±12.78 years, respectively (*P* = 0.604).

Patients group included 13 males and 11 females individuals accounting for 54.2% and 45.8%, respectively, whereas, control group included 19 males and 5 females individuals, accounting for 79.2% and 20.8 %, respectively.

With respect to gender, there was no significant difference between patients and control groups (P = 0.066), as shown in table 3.1. These findings ensure that

age and gender matching between patients and control groups which is a pre-requisite for such case control study. The lack of significant difference in mean age between patients and control groups is mandatory in such " case control study" in order to avoid bias which may result from effect of age on subsequent results.

However, these results were statistically non-significant which are consistent with many studies done by (Al-Mohaya et al). since they revealed ,that typical idiopathic FSGS is observed in person aged 18-45 years, although there is no age is exempt from the diseases(Al-Mohaya et al. 2002)).

3.2 Biochemical indices of the patient with FSGS.

Serum and urine concentrated of some biochemical indices in patients and control subjects enrolled in the present study are demonstrated in table (3.2)

Table 3.2: Biochemical indices in patients and control group

Variable	Control	Patients	*P*
Serum albumin, mean ± *SD*	4.68 ±0.89	3.97 ±0.59	0.002 € HS
Serum creatinine , mean ± *SD*	0.65 ±0.20	1.07 ±0.54	0.001 € HS
Blood urea , mean ± *SD*	27.25 ±7.95	37.79 ±8.33	0.043 € S
Cholesterol , mean ± *SD*	188.58 ±54.38	216.75 ±73.62	0.139 € NS
Triglyceride , mean ± *SD*	137.50 ±47.22	168.08 ±51.22	0.037 € S
Proteinuria , *n* (%)	0 (0.0 %)	18 (75.0 %)	<0.001 £ HS

n: number of cases; SD: standard deviation; € independent samples t-test; † Yates correction for continuity; ¥ Fischer exact test; £ Chi-square test; HS: highly significant; S: significant; NS: not significant; BU: blood urea; S.: serum.

Table 3.2 shows that the patients with (FSGS) had highly significant level of serum albumin, serum creatinine, and protein in urine more than healthy control ((3.97 mg/dl , 1.07 mg/dl , ,18% versus 4.6 mg/dl , 0.65 mg/dl , 27.25 mg/dl and 0% respectively)). (p.value 0.002 ,0.001, <0.001 respectively)

In the same table, the majority of patient with (FSGS) had significantly blood urea and triglyceride than control ((37.7 mg/dl and 168.08 mg/dl versus 27.2mg/dl and 137 mg/dl respectively)) (pvalue 0.043,0.037) other clinical characteristic such as cholesterol was statistically non-significant (p=0.139) .

These results were came in agreement with results of study done by(Someya et al.) since, they found serum urea and serum creatinine elevated in patients with FSGS (Iijima et al. 2012).

Other study done by Vega-Warner et al. found that an elevation in serum urea, serum creatinine and decreased in the serum albumin(hypoalbuminemia) (Afshinnia et al. 2013).

If Podocytes are injured, mutated, or lost, the elaborate structure of podocytes is physically altered—a process termed ‘foot process effacement’, which is found in many proteinuric kidney diseases. In some cases, once FPs are effaced (flattened down and fused), the glomerular filtration barrier is no longer intact as evidently indicated by the massive leak of proteins out of the vasculature into the urine, known as proteinuria (Mundel et.al 2010).

In fact, FSGS is a disease that leads to end stage of renal disorder(ESRD). The patient suffers from a disorder in the kidneys, which includes a group of proteins and salts that return to the blood and thus appear high levels such as urea and creatinine as well as the presence of proteins in the urine these sources have documented many studies that show the disease leads to kidney failure. Indeed,

focal segmental glomerulosclerosis is a disease involved defect in the cells of Podocytes, which is one of the main cells that cause evolution of focal segmental glomerulosclerosis (Crosson 2007).

The Disruption of the filtration slits or destruction of the Podocytes can lead to massive proteinuria, where large amounts of protein are lost from the blood. which is characterized by proteinuria leading to end-stage renal failure. also any defect in filtration will leading to problem in urea metabolism because the urea is freely filtered by the glomerulus and then passively reabsorbed in both the proximal and distal nephrons (Löwik et al. 2009).

The current results were came in agreement with previous study done by (Hayes et al.) who found that kidneys with decreased nephron mass may be more susceptible to the development of focal-segmental glomerosclerosis, Protein excretion and serum creatinine in these patients were significantly higher than in control patients (Hayes et al. 1991).

The current result directly confirm that The damage and loss of Podocytes is a primary hallmark of nephrotic syndrome. In the pursuit of targetable molecules that are involved in podocyte pathophysiology, some studies have identified B7-1 (also known as CD80) as a potential biomarker. Furthermore, B7-1 has been proposed as a podocyte-specific treatment for patients with nephrotic syndrome who have limited therapeutic options , such as those with focal segmental glomerulosclerosis (Novelli et al. 2018).

studied done by (Haikal et al) , since, they found elevated cholesterol levels in patient with focal segmental glomerulosclerosis (Haikal et al. 2016).

The increasing evidence has supported the hypothesis that lipid abnormalities contribute to both atherosclerosis and glomerulosclerosis. That are relevant to the

lipid nephrotoxicity hypothesis. They describe how inflammatory stress accompanying chronic kidney disease modifies lipid homeostasis by increasing cholesterol uptake mediated by lipoprotein receptors, inhibiting cholesterol efflux mediated by the ATP-binding cassette transporter 1 and impairing cholesterol synthesis in peripheral cells. As a result of these events, cholesterol relocates to and accumulates in renal, vascular, hepatic and possibly other tissues (Ruan et al. 2009). In conclusion The kidney is a no regenerative organ, and confirmed diagnosis of kidney disease relies on highly invasive renal biopsy. Identification of biomarkers in body fluids that can help to identify the renal pathology type will be essential for the development of noninvasive diagnostic methods. Urinary albumin, blood urea nitrogen, serum creatinine level are useful diagnostic indicators, but further information is still needed to be able to grasp the kidney disease type. For the progress of kidney disease research, it will be of crucial importance to evaluate carefully miRNA and suPAR for diagnosis .

3.3 The levels of suPAR and miRNA193a.

The comparison of the of suPAR and miRNA-193a was summarized in table below **(table 3.3)**

Table 3.3:Median levels of miRNA193a and suPAR in control and patients

Variable	Control *n* = 24	Patients *n* = 24	*P*†
miRNA-193a fold change, median (IQR)	0.375 (1.1)	2.125 (5.86)	<0.001
suPAR, median (IQR)	3671.3 (1185.62)	7873.9 (2201)	<0.001

n: number of cases; IQR: inter-quartile range; † Mann Whitney U test; HS: highly significant

The current study was compared the serum suPAR and urinary miRNA 193a in patient with FSGS by using ELISA for serum suPAR and q PCR for detection miRNA 193a ,Because these two variables(namely miRNA193a and suPAR)

proved to be not normally distributed as indicated by Kolmogorov-Smirnov test, representation was made using median and inter-quartile range (IQR) instead of mean and standard deviation as indices of central tendency and dispersion, respectively. The level of miR193a in patients and control groups was 2.125 versus 0.375 fold change, respectively and the difference was statistically highly significant (P < 0.001), being higher in patients' group than in control group, see(figure 3.1.)

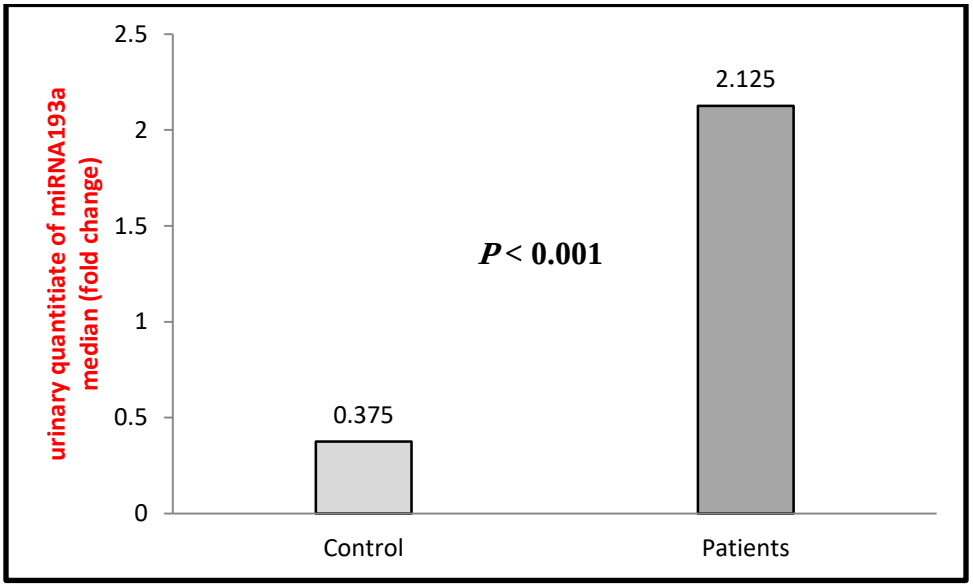


Figure 3.1: urinary quantitate of miRNA193a in control and patients groups.

Moreover, the level of suPAR was also higher in patients than in control group, 7873.9 pg/ml versus 3671.3 pg/ml, respectively; the difference was highly significant (P < 0.001), as shown in figures 3.2.

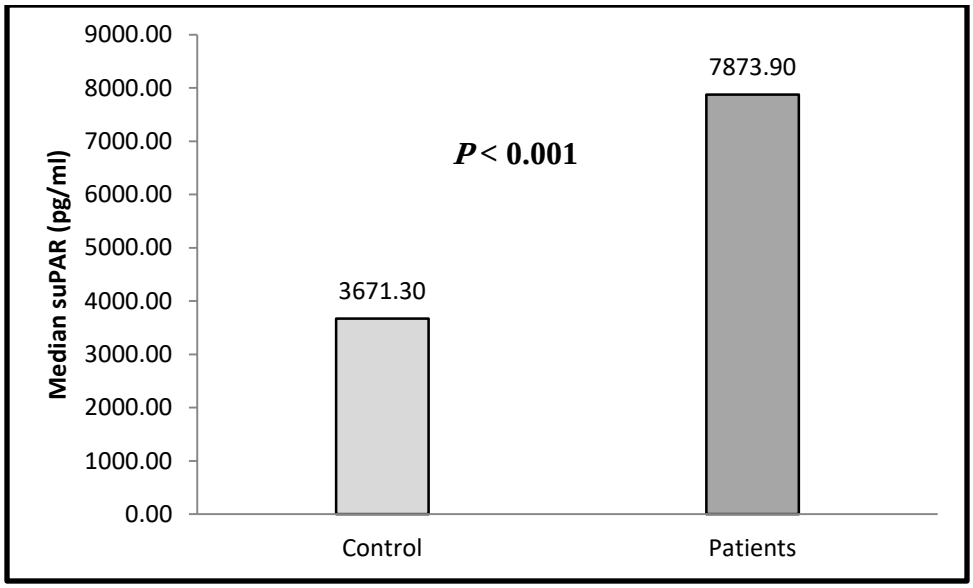


Figure 3.2: Median serum concentrate of suPAR in control and patients groups.

The present study agreement with (Peng et al.) since ,they explained that higher the serum suPAR concentration, the more severe the podocyte injury, and hence, high suPAR level might be associated with steroid resistance(Peng et al. 2015).

The finding that blood levels of suPAR were elevated in patients with recurrent FSGS has revived interest on the elusive circulating factor for FSGS. Membrane-bound uPAR on Podocytes is believed to cause proteinuria by activation of B3-integrin signaling and defect in glomerular filtration (Matsusakaet al. 2011).

The suPAR concentrations exceed the proposed threshold of 4610 pg/mL in the large majority of patients with FSGS. The causes for the accumulation of suPAR in patients at reduced glomerular filtration are incompletely understood. Urinary excretion of suPAR has been demonstrated(Huang, Liu et al. 2013).

suPAR is a circulating protein ranging from 20 to 50 kDa, depending on the degree of glycosylation and proteolytic cleavage(Savin et al. 1996).

The evidence suggests that suPAR affects the Podocyte through integrin signaling. Given the versatile roles of uPAR as a signaling orchestrator, it is tempting to speculate that the accumulation of suPAR is in the causal chain of extra renal manifestations of CKD (Soltysiak et al. 2016).

Another study done by(Halleck et al) found that FSGS patients the elevated suPAR level decreased by 32% after transplantation and stabilized at this significantly lower level, possibly due to the effect of the immunosuppression. Further observation is necessary to evaluate suPAR as a possible predictor for FSGS recurrence after transplantation(Halleck et al. 2013).

A recent study done by(Ding et.al) since they that highlighted the immune-mediated pathogenicity of primary FSGS, especially in patients who are sensitive to steroids(Ding et al. 2014).

The initial steroid responsiveness was related to higher suPAR levels (≥3400 pg/ml), which suggested that serum suPAR elevation may possibly underlie the pathogenesis of some patients with FSGS. suPAR level assays may help determine a therapeutic regimen for patients with FSGS (Sever et al. 2013).

Another study identifies suPAR as a circulating, predictor FSGS factor that is elevated in the serum with primary FSGS patients. Which activation of β3 integrin on Podocyte foot processes this mechanism of injury caused by high suPAR blood concentrations.(Bock et al. 2013).

the major cause of the elevated suPAR levels of our patients with primary FSGS. uPAR, the glycosylphosphatidylinositol (GPI)-anchored protein with three domains (DI, DII, and DIII), is expressed on several different cell types, including neutrophils, monocytes, macrophages, activated T-lymphocytes, endothelial cells

and kidney podocytes . It could be released to plasma as suPAR after being cleaved of the GPI anchor, and it is also susceptible to cleavage at the linker region between DI and DII, so both the whole receptor and various segments of it are found free in the serum and are all called suPAR (Halleck et al. 2013).

In addition to regulation of proteolysis, suPAR initiates signaling transduction in cooperation with other trans membrane proteins, such as integrins, caveolin and G-protein-coupled receptors, which promotes cell proliferation, invasion, motility and survival. However, the pathogenic domain or part of the suPAR molecule in FSGS is not fully elucidated; it might be a specific domain, or a special form of glycosylation or phosphorylation of this interesting molecule. Although our results suggest that suPAR might play an important role in the pathogenesis of primary FSGS, there is still an overlap of urinary suPAR levels between patients with primary FSGS and patients with secondary FSGS and other glomerular diseases. In addition, various forms of the suPAR molecule exist in both plasma and urines in physiological conditions(Huang et al. 2014).

Regarding the micro RNA play role in pathogenesis of FSGS due to defect in podocyte that founded in glomerular basement membrane are are critical in the maintenance of structure and function of the glomerular filtration barrier. The Recent studies revealed altered expression of miRNAs in the kidneys during the progression of acute kidney injury (AKI) and chronic kidney disease (CKD) in humans and experimental rodent models (Ichii 2018).

These results were came in agreement with results done by (Gebeshuber et. al)since they found that transgenic expression of the microRNA miR-193a in mice rapidly induces FSGS with extensive podocyte foot process effacement (Gebeshuber et al. 2013).

The cause of the majority of cases of FSGS remains elusive(D'Agati et al. 2011).

As FSGS targets primarily the podocytes, deeper insights into the stabilization mechanisms of podocytes are important, and the question of a potential role of regulatory miRNAs arises. It was previously shown that global podocyte-specific ablation of miRNAs by knockout of the miRNA-processing enzymes Dicer or Drosha leads to proteinuria and glomerulosclerosis(Shi et al. 2008) ,(Zhdanova et al. 2011).

However, the role of specific miRNA , to the best of our knowledge, has not been addressed. In a study done by Menke et al. they revealed that mechanistic insight into the molecular pathogenesis of glomerular damage induced by dysregulation of a single miRNA. Inducible up-regulation of miR-193a in transgenic mice led to rapidly progressing FSGS and death from renal failure within 12 weeks. Mechanistically, miR-193a binds to and represses *WT1*, a gene that is essential for the development and maintenance of normal podocytes and glomeruli (Guo et al. 2002),(Chau et al. 2011).

The study were done by (Chandrasekaran et. al) found that miRNA is being increasingly found to have important regulatory roles in the development, physiology, and maintenance of adult-kidney microstructure. Though expression profiles of miRNAs in various renal diseases have already been examined, further studies are needed for a thorough understanding of the roles of miRNA in renal pathophysiology. miRNAs form valuable tools for diagnosis of several kidney diseases. This review provides an overview on the crucial roles that miRNAs have in renal function and diseases. The involvement of miRNAs in various kidney diseases/pathogenesis (Chandrasekaran et al. 2012).

The miRNA plays a key role in preventing protein synthesis through DNA work, and inhibits the translation process in an early step, possibly at the beginning of the translation, which eventually follows the mRNA decay (Li et al. 2013).

Through previous studies have shown that the miRNA affect the cellular metabolism and have a direct or indirect role in the impact on the physiology of disease for a range of diseases, the most important kidney disease through its impact on Nephron and then can be indicative of its presence in the diagnosis of diseases especially FSGS(Ventura et al. 2008),(Wei et al. 2013).

In addition, the miRNA play an important role in the regulation of tubular and glomerular damage and proteinuria due to podocyte specific deletion of dicer (Duisters et al. 2009).

The miRNA play important in regulate gene expression at posttranscriptional level and then play role in different cellular function and physiological activities (Wei et al. 2013). More research suggests the present miRNA 193a has a direct relationship to the early detection of FSGS in a patients through its association with oxygen deficiency hypoxia (Fogo 2015) .

In addition to the mentioned, MicroRNA involved in the processes of cellular broad and important in the physiology of the pathogenesis of many types of cells, especially the podocyte Through Dicer targeted deletion. The Dicer is the important enzyme in the synthesis or biogenesis of miRNA Thus resulting in it appear the protein in the urine (proteinuria), podocyte injury, glomerulosclerosis and finally leading to end stage of the renal disorder (ESRD).(Shi, Yu et al. 2008)

The miRNA also play roles in a mesangial and endothelial cell which are two glomerular cell types that interact with Podocytes then leading to defect in the

work of kidney and development the pathogenesis of FSGS in patients (Pollak 2002).

3.3.1- Correlation between suPAR and miRNA 193a:

According to our knowledge, there is no study performed the correlation between suPAR and miRNA 193a , the current study show that there are appositive correlation. Added to that, the bi-variate Spearman correlation was positive and highly significant (r = 0.502; P < 0.001); however, this correlation was able to predict only 5.1% of variation in unites suPAR versus units miRNA193a, since the R2 value was extremely low (0.051) (see figure 3.3)

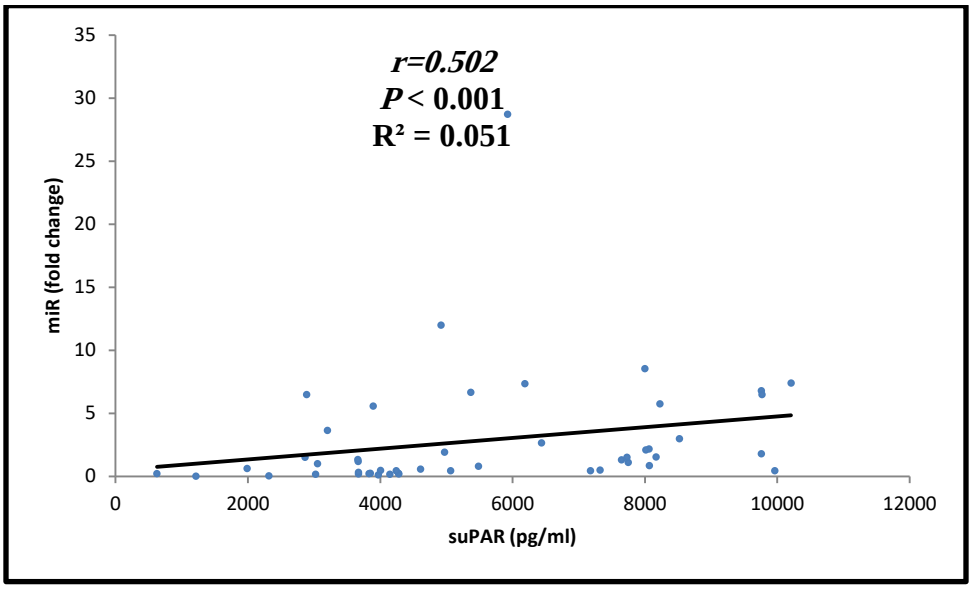


Figure 3.3: Correlation between miRNA193a and suPAR in study groups.

One of the possible mechanisms for appearance of suPAR in blood results from damage of podocytes that mostly seen in patients with FSGS. This point give highlight about the size of damage , as the current study show significant positive correlation ,thus , damage is sever , lead to high level of suPAR which confirmed

with elevation of miRNA-193a in urine. This positive correlation could be explained according to scientific pathological process of damage in FSGS.

Keeping in mind this the first study in Iraq conducted to find such correlation between suPAR and miRNA 193a in patient with FSGS . unfortunately , there was no previous study mentioned the correlation between suPAR and miRNA193a in pathogenesis of FSGS.

Such as what is known that miRNA-193a is a genome located within the podocyte cell When the crash of this cell will significantly deteriorate, The reason for selecting MicroRNA is its stable presence in the urine and stays for long periods. It can be determined in very precise quantities and thus support the hypothesis of research. In addition, the SuPAR protein receptor proves the hypothesis of research on the presence of miRNA 193a, which is also an easy work test measured by ELISA technique by sampling the blood from the patient with FSGS, miRNA193a is produces from the crash of the podocyte cells and this crash is evidence of the disease. In summation we may use a diagnosis and we will explain in the privacy and sensitivity section.

3.4 Correlations of miRNA193a and suPAR to laboratory findings.

Table 3.4 shows the summary the correlations of suPAR and miRNA193a to clinical and laboratory parameters:

Table 3.4: Correlations of suPAR and miRNA193a to laboratory parameters.

	suPAR		miR193a	
Characteristic	*r*	*P*	*r*	*P*
Age	-0.056	0.703	-0.094	0.525
Gender	0.213	0.147	-0.035	0.812
Serum albumin	-0.412	0.004	-0.016	0.915
Serum creatinine	0.479	0.001	0.070	0.634
Blood urea	0.347	0.016	-0.034	0.820
Cholesterol	0.179	0.224	0.128	0.386
Triglyceride	0.301	0.038	-0.047	0.750
Urinary protein	0.598	<0.001	0.224	0.125

According to table (3.4) the present study demonstered that there were highly significant positive correlation between suPAR and serum creatinine (r = 0.479; P = 0.001) ,urinary protein (r = 0.589; P <0.001).and highly significant negative correlation with serum albumin (r = - 0.412; P = 0.004). While, suPAR show significant positive correlation with serum triglyceride (r = 0.301; P = 0.038) and blood urea (r = 0.347; P = 0.016).

However regarding to mi-RNA193a present study found that there were no-significant correlation between such marker and any of biochemical finding. The absent of significant correlation in this study may be due to the small number of sample . thus , another study based on large sample size is needed to solidify present study.

This result agree with a study done by Bajpai et al . since they were propose that The urinary suPAR-to-creatinine ratio significantly correlated with proteinuria (Sinha et al. 2014).

Also the creatinine is derives from creatine degradation with a weight of 113 Da(Colls 1896, Stevens, Coresh et al. 2006)]. It is freely filtered but is not reabsorbed or metabolized however a significant percentage of creatinine in the urine derives from proximal tubular secretion (Shemesh, Golbetz et al. 1985)One of the requirements for utilizing estimating equations based on SCr is stable kidney function.so that any defect in the function of kidney leading to problem in the level of creatinine in blood (Levey et al. 2014).

In this study there was significant correlation with blood urea The blood urea nitrogen (BUN) test measures the amount of nitrogenous waste in the patient's blood. If a patient has high levels of waste products in the blood, it indicates that the kidneys are not able to filter the blood. Healthy individuals usually have 7-20 milligrams of waste per deciliter of blood. Elevated levels indicate kidney disease (Abbate et al. 2006)**.**

the present study came agreement with previous study done by Marshall et al. since they found the urokinase receptor has been shown to play a direct role in regulating podocyte foot process structure and function and plays a critical role in maintaining the selectively of glomerular permeability (Smith and Marshall 2010). If podocytes are injured, mutated, or lost, the elaborate structure of podocytes is physically altered—a process termed ‘foot process effacement’, which is found in many proteinuric kidney diseases. In some cases, once FPs are effaced (flattened down and fused), the glomerular filtration barrier is no longer intact as evidently indicated by the massive leak of proteins out of the vasculature into the urine, known as proteinuria (Wei et al. 2008), (Mundel et.al 2010).

In FSGS suPAR recognizes the podocyte actin cytoskeleton causing podocyte foot process effacement, which leads to proteinuria, causing secondary hypoalbuminemia (Chang et al. 2012).

The present study came in disagree with study done by Bajpai et al, Mao et al ,since ,they did not find correlations between suPAR levels and proteinuria or serum albumin levels (Sinha et al. 2014),(Peng et al. 2015).

They attributed this to either the presence of circulating factors other than suPAR or the presence of a specific suPAR fragment that is not detected by ELISA, which can cause proteinuria (Sever et al. 2013).

another study done by(Rasmussen et. al) suggest that beside the risk associated with the traditional risk factors, such as total cholesterol and LDL, suPAR may reflect a different pathogenesis of a more inflammatory nature(Rasmussen et al. 2016).

Also study done by (Greenberg et al.) since they found that Cholesterol aeroembolism with FSGS should be considered in the differential diagnosis of nephrotic syndrome in elderly patients with advanced atherosclerosis (Greenberg et al. 1997).

Persistent severe albuminuria reduces the serum albumin level and leads to a compensatory increase of albumin synthesis in the liver, but lipoprotein synthesis is also increased simultaneously and hyperlipidemia occurs. In addition, the enzyme catabolizing lipoproteins is excreted in the urine; this exacerbates hyperlipidemia. The resulting increased uptake of oxidized LDL by glomerular mesangial cells causes mesangial hyperplasia and leads to glomerular sclerosis Furthermore, uptake of lipid droplets by renal tubular cells causes tubulointerstitial damage. Moreover, phagocytosis of lipids by macrophages in the blood vessel walls leads to progression of arteriosclerosis, which also worsens renal damage. Thus, elevated lipid levels accelerate renal dysfunction by affecting the renal

tubules and blood vessels in addition to the glomeruli. (Diamond and Karnovsky 1988). Moreover ,This result agree with a study done by(Xiao et al) ,since they found no correlation was found between miRNA and clinical parameters including creatinine, eGFR, proteinuria, serum albumin, and treatment type. (Xiao et al. 2018).

3.5-Sensitivity and specifity of suPAR and miRNA-193a.

Table 3.5 show that the sensitivity and specifity for suPAR were 100% and 95.8% respectively with AUROC was 0.998 . for miRNA-193a the sensitivity and specifity were 100% and 50% respectively with AUROC was 0.826, both of the studied parameter were high significant (all p<0.001).

Table 3.5: Receiver operator characteristic (ROC) curve parameters

Variable	Cutoff	AUC	95% CI	*P*	Sensitivity	Specificity
miRNA193a	> 0.31 fold change	0.826	0.690-0.920	<0.001	100%	50%
suPAR	> 4610.15	0.998	0.923-1.000	<0.001	100 %	95.8 %
suPAR versus miRNA193a	-------	0.172	----------	0.003	Same	Better

This study demonstrated that suPAR have cutoff value that yield the highly specifity and sensitivity thus , is marker might be involved in the pathogenesis of FSGS and giving important diagnostic value .

The present study agreement with previous study done by (Saleem 2018) since he demonstrated have high specifity and sensitivity ., while the miRNA193a is less specificity about 50% while the sensitivity were 100%.so the suPAR have highly diagnosis value over than miRNA193a for diagnosis FSGS. that soluble urokinase receptor might be the most likely causative circulating factor for primary

FSGS. In additional the high serum suPAR concentration is associated with more severe podocyte injury leading to more resistance to steroid therapy(Peng et al. 2015).

Previous studies have shown that specifity and sensitivity for the suPAR to development of FSGS are very high . These studies are consistent with this study (Segarra et al.) That revealed soluble urokinase-type plasminogen activator receptor (suPAR) levels could be useful for distinguishing idiopathic focal segmental glomerulosclerosis (FSGS) from other glomerulopathies that cause nephrotic syndrome, but these data have not been confirmed in independent studies. The objective of this study is to analyses whether circulating levels of suPAR are useful for identifying primary kidney disease in patients with nephrotic syndrome secondary to FSGS(Segarra et al. 2014).

The results of this study disagreement with a study done by (Segarra et al.) they explain suPAR level >3531pg/ml could have a high specificity (but a low sensitivity) in the diagnosis of FSGS (Segarra et al. 2014).

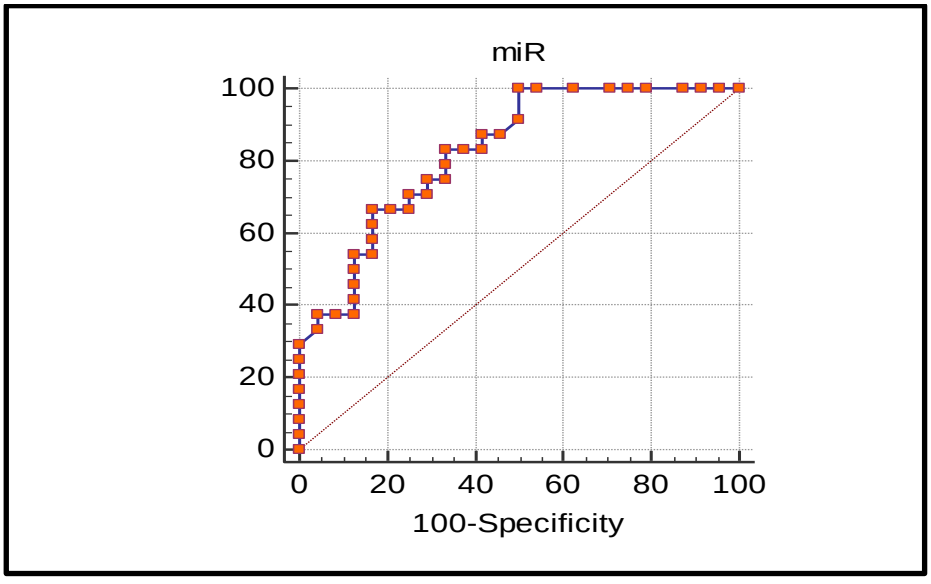


Figure 3.4: Receiver operator characteristic (ROC) curve of miRNA-193a cutoff value

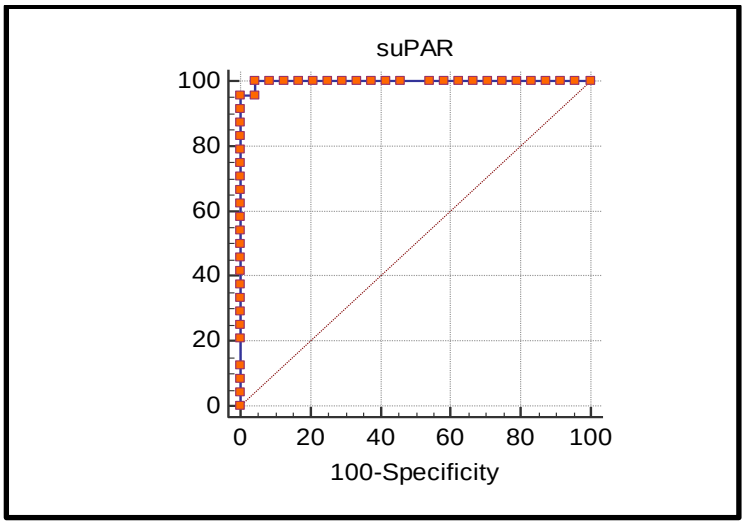


Figure 3.5: Receiver operator characteristic (ROC) curve of suPAR cutoff value.

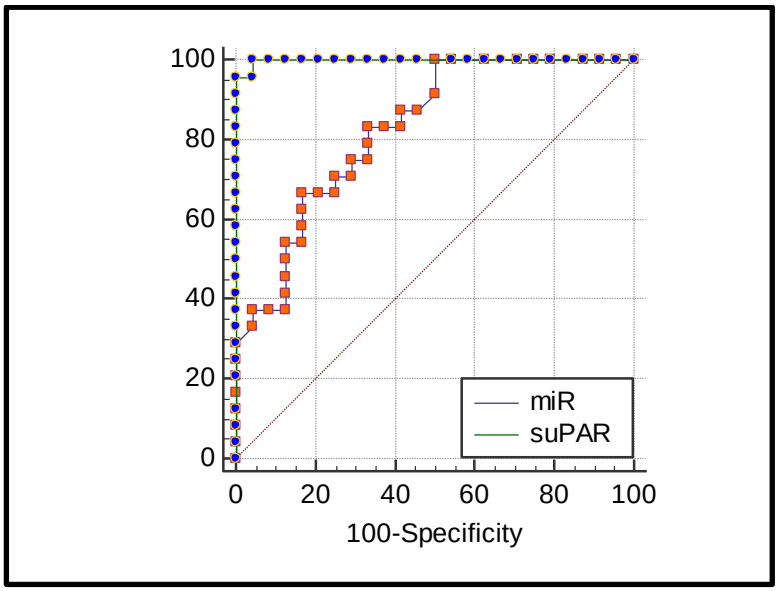


Figure 3.6: Receiver operator characteristic (ROC) curve comparing miRNA193a to suPAR.

Indeed, the high specifity and sensitivity for the suPAR through the destruction process of podocyte that play an important role in glomerular function. Together with endothelial cells of the glomerular capillary loop and the glomerular basement membrane they form a filtration barrier. Podocytes cooperate with meningeal cells to support the structure and function of the glomerulus. With

regard to its structure, the podocyte can be divided into three structural and functional segments: cell body, major processes, and foot processes, the latter playing a crucial role in the pathogenesis of proteinuria. Podocytes are end-differentiated cells with specific phenotypic features associated with their function (Peng et al. 2015).

When shatter, special material called a circulating factor such as soluble urokinase plasminogen activator receptor(suPAR), cardiotropin-like cytokine-1(CLC-1),vitronectin and integrin. Which will be introduced through the blood directly and therefore can be shown to the emergence of high rates due to the disease of focal segmental glomerulosclerosis(Sever et al. 2013).

the soluble form of urokinase plasminogen-type activator receptor, has been reported to be elevated in a number of diseases including cancer and infection in a nonspecific manner. However, its underlying mechanism of action in these conditions and its clinical value either in diagnosis or prognosis is still far from clear. Wei *et al.* reported the molecular identity of a putative permeability factor in FSGS(Wei et al. 2011).

A biomarker for clinical use needs good sensitivity and specificity and good positive and negative predictive value. However, previous studies on suPAR had many problems in the study designs, sample collection, and statistical analysis techniques. Most studies on suPAR were conducted in a retrospective design and the selection of healthy controls was not matched for age, sex, and other parameters influencing suPAR levels. With regard to statistics, most studies did not perform multiple logistic regression analysis to find an independent predictor and receiver operating characteristics (ROC) curve analysis to calculate sensitivity and specificity, an essential prerequisite to be a biomarker, but simply presented the

differences of suPAR levels among groups. Furthermore, some studies did not present the mean ± standard deviation (SD), hampering the meta-analysis of several suPAR studies. If there are many factors influencing suPAR levels, an individual patient data meta-analysis and propensity score matching would be important statistical methods to elucidate whether suPAR could be a reliable surrogate biomarker in this field. The results of suPAR may be cross-linked with other disease ,and also suPAR may be to give normal or decreased results in FSGS because the measure total serum suPAR consist from three fragment including (D1,D11 and D111) So far, it has not been known who is responsible for the development of the disease (Mousa et al. 2018).

These results is agreement with the study done by(Huang et al.) that found to evaluate the diagnostic values of urinary exosomal miR-193a for primary FSGS, ROC curves were generated to discriminate primary FSGS from MCD in children. the found an area under the ROC curve (AUC) of 0.85 (95% confidence interval [CI] 0.63–1.07)). A ROC analysis identified an optimal threshold of urinary exosomal miR-193a for the diagnosis of FSGS at 530, with a high sensitivity of 75% and a high specificity of 80%. This finding indicated that urinary exosomal miR-193a may be a good index for the differentiation between primary FSGS and MCD in children. levels of urinary exosomal miR-193a were significantly higher in children with primary FSGS than those in children with MCD (Huang et al. 2017).

The role of specific miRNAs in normal renal development and physiology, but also the initiation and the progression of the interstitial fibrosis that underlies progressive forms of chronic kidney disease. It follows, that miRNAs detected in either plasma o urine, the two fluidic compartments directly affected by renal processing, may be mechanistically plausible, rational biomarkers for diverse

forms of kidney diseases. In fact, miRNA associations found in observational human studies may offer a unique opportunity to “reverse translate” such findings into animal studies, which provide mechanistic insights into novel therapeutics that are tested in rigorous interventional clinical trials in humans..miRNAs are endogenously expressed in the kidney and several have been found to be up- or downregulated in renal tissue in various kidney diseases (Kato et al. 2009), (Chandrasekaran et al. 2012).

A recent elegant study showed that miR-193a is up-regulated significantly in podocytes in FSGS, where it directly targets the expression of WT1, a key transcription factor for podocyte differentiation and health. Surprisingly, extracellular miRNAs are abundant in blood and other biological fluids,(Creemers et al. 2012) where they are shielded from nucleases by being packaged in lipid microparticles (such as in exosomes and microvesicles) or by association with protein (such as Argonaute 2) and lipoprotein (such as high-density lipoprotein) complexes. The remarkable stability of circulating miRNAs has made them valuable for use as novel biomarkers in multiple human diseases. other Study done by Arce et al. that they found to identify a number of unique miRNA signatures that are associated with human kidney diseases, including FSGS (Kato et al. 2009).

miRNAs have also been used as biomarkers both in serum and urine to assess FSGS disease activity. In one study, researchers found elevated plasma has-miR-125b-5p, has-miR-186-50 and has-miR-193a-3p in patients with FSGS with area under curve (AUC) of 0.88, 0.78, and 0.91, respectively . Patients in remission had lower has-miR-125b-5p and has-miR-186-5p concentrations . These miRNA levels remained unchanged in patients that did not achieve remission (Zhang et al. 2015).

Chapter Four

Conclusion & Recommendation

Conclusions

1. The current results confirm that the mean age of prevalence of focal segmental glomerulosclerosis in population was 28 years.
2. The present finding indicated that both soluble urokinase plasminogen activator receptor and miRNA-193a elevated in patient focal segmental glomerulosclerosis(FSGS) than the control which confirm that suPAR and miRNA-193a play a critical role of the pathogenesis of FSGS.
3. On the basis of the current study the ROC analysis revealed that the sensitivity and specific for suPAR were 100% and 95.8% respectively while it was 100% and 50% respectively for the miRNA-193a .therefore ,by comparison , suPAR is significantly more accurate for diagnosis of the disease thus it consider as non-invasive diagnostic marker for FSGS.
4. The analyses conducted in this study showed that the concentration of suPAR significant correlated with serum albumin (negative correlation) ,serum creatinine (positive correlation), blood urea (negative correlation) and both serum triglyceride and protein in urine (positive correlation).
5. The present result show that no significant association between miRNA-193a and all biochemical test.
6. Association between suPAR and miRNA-193a .

Recommendations:

1-further study based on large sample size are needed to make large data base for determined the role of suPAR & miRNA-193a as a biomarker for diagnosis and fallowing FSGS in patient .

2-study the combined role of other biomarker , not included in the present study , like CLC-1 , vitronectin and integrin in relation to FSGS.

3- polymerase chain reaction (PCR) could be apple to detect the expression level of suPAR as a prognostic marker for FSGS patient.

4-afollow up- study based on reasonable no of patient in order to find prognostic importance of these biomarker in response to treatment.

5- another study design to find out diagnostic value of these biomarker in differentiation between FSGS and MCKD.

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Appendix

Appendences

Appendix 1: patient datasheet

Case number			
Name			
age			
Sex	male		female
Biochemical testing			
Blood urea			
Serum creatinine			
Serum albumin			
Protein in urine			
cholesterol			
Triglyceride			
biopsy			
NOTE			

أعلى في المرضى منه في المجموعة الضابطة ، ٧٨٧٣.٩ (٢٢٠١) مقابل ٣٦٧١.٣ (١١٨٥.٦٢) ، على التوالي ؛ كان الفرق معنويا للغاية (P <٠.٠٠١)

لدراسة الدور المحتمل لكل من ميرنا ١٩٣ أ و suPAR في تشخيص ومتابعة المرضى الذين يعانون من التهاب كبيبات الكلى ، كشفت خاصية المشغل المتلقي أن قيمة قطع ميرنا١٩٣ أ كانت 0.31 <أضعاف التغيير مع حساسية ١٠٠٪ وخصوصية ٥٠٪ .كانت AUC عبارة عن ٠.٨٢٦ (فاصل الثقة ٩٥٪: ٠.٦٩٠-٠.٩٢٠) وبالتالي دقة ٨٢.٦٪ ومستوى الأهمية (P <0.001).من ناحية أخرى ، كانت قيمة قطع suPAR> 4610.15 مع حساسية ١٠٠٪ وخصوصية ٩٥.٨٣٪ .تكون) AUC 0.998 فاصل الثقة ٩٥٪: ٠.٩٢٣-١.٠٠٠) وبالتالي دقة ٩٩.٨٪ ومستوى الأهمية.(P <0.001).

في الختام ، فإن suPAR أكثر دقة بشكل ملحوظ ، وأيضا أكثر تحديدا لتشخيص المريض مع FSGSبدلا من الخزعة.

التجلط الكبيبي القطعي البؤري الابتدائي هو شكل تدريجي من مرض الكُلى الكلوي الناجم عن تصلب / تندب الكبيبات. الخزعة الكلوية هي الأداة النهائية لتشخيص FSGS التي تعتبر تقنية ضاره وتتطلب التدخل الجراحي.

أهداف الدراسة الحالية هي التحقيق فيما إذا كان المرضى الذين يعانون من تجلط الدم القطعي البؤري (FSGS) لديهم خصائص متميّزة suPAR و miRNA-193a التي يمكن أن تؤدي إلى تطور محتمل للعلامات الحيوية التشخيصية للمرض. وأخيرا تحديد أي منها لديهم حساسية وخصوصية أعلى باستخدام متطوعين أصحاء كمجموعة سيطرة. ولتحقيق هذا الهدف تم جمع عينات دم وكذلك الادرار من ٢٤ مريض عراقي من بينهم (١٣ ذكور و ١١ إناث) مع FSGS الأولية ، والذين حضروا للعيادة الاستشارية لأمراض الكلى في مستشفى الديوانية التعليمي في الفترة ما بين ١ يناير ٢٠١٨ الى ١٠ مايو ٢٠١٨ تحت إشراف أخصائي أمراض الكلى. في هذه الدراسة تم تشخيص المرضى مع FSGS وفقا للتقرير النسيجي من الكلى (خزعة) بالإضافة إلى المعلومات عن كل حالة جمعت من المريض.

بالإضافة إلى ذلك ، تم تضمين ٢٤ متطوعًا أصحاء كمجموعة مراقبة. تم جمع عينات الدم لاستخدامها في اختبار فحص المواد المناعية المرتبط بالإنزيم لتحديد تركيز مستقبل المنشط البلازمينوجين القابل للذوبان في مصل الدم في حين تم جمع عينات الادرار من (منتصف السريان) لاستخدامها في تفاعل البلمرة المتسلسل الكمي لأحماض الريبونوكليوتيد للدراسة تم تلخيص ميرنا ١٩٣a في المريض مع عرض وتحليلها باستخدام حزمة إحصائية للعلوم الاجتماعية (SPSS الإصدار ٢٣)

أظهرت الدراسة الحالية أن متوسط الزلال المصلي كان أقل بشكل ملحوظ في مجموعة المرضى مقارنة مع المجموعة الضابطة ، ٣.٩٧ ± ٠.٥٩ جم / ديسيلتر مقابل ٤.٦٨ ± ٠.٨٩ جرام / ديسيلتر (P = 0.002) ، بالإضافة إلى متوسط الكريتيانين في المصل كان اليوريا في الدم أكبر بشكل ملحوظ في مجموعة المرضى مقارنة مع المجموعة الضابطة ، ١.٠٧ ± ٠.٥٤ ملغم / ديسيلتر مقابل ٠.٦٥ ± ٠.٢٠ ملغ / ديسيلتر و ٣٧.٧٩ ± ٨.٣٣ ملغم / ديسيلتر مقابل ٢٧.٢٥ ± ٧٠.٩٥ ملغم / ديسيلتر ، على التوالي (P <٠.٠٥) وسريريا كان البروتين القابل للكشف في البول (بروتينية) يقتصر على المرضى الذين يعانون من التهاب كبيبات الكلى. كان مستوى miR193a في المرضى والمجموعات الضابطة ٢.١٢٥ (٥.٨٦) أضعاف التغيير مقابل ٠.٣٧٥ (١.١) تغيير التغير ، والفرق كان معنويا إحصائيا (P <٠.٠٠١) ، كونه أعلى في مجموعة المرضى من المجموعة الضابطة ، علاوة على ذلك ، كان مستوى suPAR أيضا

الخلاصة

وزارة التعليم العالي
والبحث العلمي
جامعه القادسية
كليه الطب

تقييم كفاءة suPAR في المصل و miRNA-193a في الادرار كمؤشرات حيوية لتشخيص تصلب الكبيبات القطعي البؤري الابتدائي

رسالة مقدمه الى
مجلس كلية الطب ـجامعة القادسية
كجزء من متطلبات نيل درجة الماجستير
في علم الاحياء المجهرية الطبية

من قبل

علي رضا عبد الامير شير علي

بكالوريوس في تقنيات التحليلات المرضية/بغداد (٢٠١٣)

بأشراف

أ.م.د.أبراهيم عبد المجيد مصطفى التميمي

١٤٤٠ هـ

2018 م