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Title: miR-148b differentiates Tubular atrophy/Interstitial fibrosis histopathological stages in IgA nephropathy

Authors: Santosh Kumar[1], C Priscilla[1], Sreejith Parameswaran[2], Deepak Gopal Shewade[3], Pragasam Viswanathan[4], Rajesh Nachiappa Ganesh[1]

[1] Department of Pathology, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India - 605006

[2] Department of Nephrology, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India - 605006

[3] Department of Pharmacology, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India – 605006

[4]Department of Bio Sciences, School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore, Tamilnadu, India – 632014

Corresponding author

Rajesh Ganesh Nachiappa

Additional Professor

Department of Pathology

Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India

E-mail: drngrajesh@gmail.com

Phone: +91-9791976021

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Abstract

IgA nephropathy (IgAN) is one of the most common forms of glomerular disease. It is diagnosed by the dominant or co-dominant IgA deposition in the mesangial region by histopathological examination of kidney biopsy. Kidney biopsy has its own complication and not performed frequently. microRNA (miRNA) is a small RNA, which plays an important role at the post transcriptional level by downregulating mRNAs. We have tried to establish a miRNA based biomarker for IgAN.

We quantified miR-148b and let-7b from plasma in IgAN patients and healthy controls. Logistic regression models and receiver operating curve analysis used to analyze the miRNAs quantity and Oxford MEST-C scoring parameters (M- Mesangial hypercellularity, E- Endocapillary hypercellularity, S- Segmental glomerulosclerosis, T- Tubular atrophy/Interstitial fibrosis, C- Crescents).

miR-148b and let-7b levels in IgAN were found to be higher by 2.9 and 5.48 times than the healthy controls, respectively. let-7b was positively correlated with complement C3 levels. Similarly, miR-148b was positively correlated with estimated glomerular filtration rate (eGFR) and negatively correlated with S, T, and blood pressure (BP). The sensitivity, specificity, and area under the curve (AUC) of receiver operating characteristic (ROC) for miR-148b against T were 0.87, 0.77, and 0.85, respectively. The threshold value of miR-148b concentration was found to be 8479 to differentiate the severe condition of IgAN. Furthermore, the decrease in miR-148b concentration at a threshold point indicated the progression of the severity of the IgAN. It can also be used to predict the IgAN at an earlier stage.

Keywords: IgAN, miR-148b, let-7b, Tubular atrophy, Interstitial fibrosis, MEST-C

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Introduction

IgA nephropathy(IgAN) is a slowly progressive kidney disease whose pathogenesis is not completely known[1,2]. It is one of the most common glomerular disease worldwide, where around 25 percent of IgAN patients reach to end stage kidney disease within twenty years of diagnosis[3]. It is diagnosed by histopathological examination of kidney biopsy with dominant or co-dominant deposition of IgA molecules at the mesangial region[4]. The IgA molecules deposited at the mesangial area were found of type IgA1 and galactose deficient[5].

Kidney biopsy is an invasive procedure and has its own complications. It is not frequently repeated as well. Delayed kidney biopsy is another drawback where the kidney occurs a significant damage by the time IgAN is diagnosed. The characteristics of IgAN and kidney biopsy practice seemed to be different in different parts of the world and it is not yet clear that it is the same disease worldwide[6–9]. The IgA nephropathy classification group, in 2017, proposed a modified histopathological scoring system known as Oxford MEST-C score (M- Mesangial hypercellularity, E- Endocapillary hypercellularity, S- Segmental glomerulosclerosis, T- Tubular atrophy/Interstitial fibrosis, C- Crescents)[10]. MEST-C score with cross-sectional clinical data at biopsy has shortened the time-frame to accurately predict patients at risk of adverse kidney outcome[11]. Among all the histologic parameters of MEST-C, T and C can hold the accurate predictive value for the adverse outcome of the disease[12]**.** T0 reflects the absence, T1 reflects 25% to 50%, and T2 reflects 50% onwards of fibrosis/tubular atrophy involvement. The same way, C0 represents no crescent, C1 up to 25%, and C2 reflects crescents in more than 25% of glomeruli.

miRNAs are small and approximately 22 nucleotides in length[13]. It is highly conserved, found in the intronic region, and downregulates the mRNA post-transcriptionally[14]. We have selected miR-148b and let-7b as the proposed predictive markers based on a microarray based

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dataset and published literatures[15,16]. miR-148b and let-7b have been associated with aberrant-glycosylation process and found regulating the enzyme core 1 β 1,3 galactosyltransferase 1(C1GALT1) and the enzyme N-acetylgalactosaminyl transferase 2 (GALNT2) respectively[17.18]. Furthermore, the aberrant glycosylation process at the hinge region of the IgA1 molecule has been associated with the pathogenesis of IgAN[19]. In a previous study, it was shown that the anti-glycosylation process changed the miR-148b and let-7b expressions in IgAN patients[18]. It is considered that miR-148b and let-7b might be playing a role in the pathogenesis of IgAN and have the potential to be used as a diagnostic and prognostic marker for the prediction of IgAN. In this study, we have tried to find the diagnostic and prognostic importance of miR-148b and let-7b for IgAN.

Material and methods

Study participant selection

All consecutive native biopsies, reported as primary IgA nephropathy, were considered for the study, while secondary causes of IgA nephropathy, patients with systemic lupus erythematosus (SLE), Henoch Schonlein purpura (HSP), other autoimmune disease, kidney carcinoma, human immuno deficiency virus infection, hepatitis, diabetes mellitus and infection related glomerulonephritis were excluded. The participants were in the age group of 15 to 70 years. Institute human ethics committee approval was taken for the study. We recruited 30 IgAN patients and 15 healthy persons (age and sex matched) as study participants after taking their informed written consent following the inclusion and exclusion criteria.

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Sample collection

Five ml blood was collected into EDTA tubes after taking the informed written consent from the study participants. The sample was processed within one hour of collection. Plasma was collected after centrifugation at 3000 g value for five minutes at four degree temperature. Plasma was stored immediately in aliquots at -80^0 C until further processing.

microRNA quantification

microRNA (miRNA) quantification performed in four experimental stages. The first stage was performed as miRNA isolation from the plasma sample. The second step was to check the quality of the isolated miRNA. Fluorometer (Qubit 3.0, ThermoFisher) used for this experiment. The third stage was performed as cDNA conversion from the isolated miRNA. The fourth and final stage was performed for the amplification of the target miRNA to see the expression. Real time PCR (Quant Studio 3, ThermoFisher) instrument used for cDNA conversion and miRNA expression. The necessary chemicals, reagents, primers, and probes were procured from Invitrogen, ThermoFisher, and Helini Biomolecules. The mean expression value method used for the normalization of miRNAs. Standard curve method used for the absolute quantification and calculation of copy number of miRNAs. We followed a previously established method for miRNA quantification in our laboratory that was published earlier this year[20].

Histopathological analysis

Kidney biopsy of IgAN patients was examined and interpreted by histopathological examination through immunofluorescence light microscopy. MEST-C scoring under Oxford classification was used and kidney biopsy findings were documented.

 doi:10.20944/preprints202006.0049.v1

Statistical Analysis

Shapiro-Wilk normality test performed to check the data distribution. Wilcoxon's rank sum test and t-test were performed for probability distinction according to the data distribution. Logistic regression method used for the predictive features of miRNAs against MEST-C score. Area under curve (AUC) of receiver operating characteristic(ROC), Sensitivity (SE), Specificity (SP), Akaike information criteria (AIC) and Bayes information criteria (BIC) were used for the best fit model selection[21]. The cutoff value for AUC, SE, and SP was set at 0.70. Correlation analysis was performed by Spearman's rank method. The cutoff value for the correlation coeffici ent was fixed at 0.33 at p value < 0.05. Mean values were given with standard deviation (SD) and median values with inter quartile range (IQR). The receiver operating characteristic curve was m ade by pROC package of R[22]. All the statistical analysis performed using R version 3.6.2[23].

Ethical approval

"All procedures performed in this study involving human participants were in accordance with the ethical standards of the institute research and human ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards." The study has been started after the approval of the institute human ethics committee (JIP/IEC/SC/2015/19/785). Informed written consent was taken from all individual participants included in the study.

Results

The mean age group of IgAN participants was 29 (9.63) years. About 47 % IgAN patients were male and 53 % female. The average systolic and diastolic blood pressures of the patients were 122 (110 – 142) and 82 (70 – 92.50) mmHg, respectively, and found to be in stage-1 hypertension state [24]. The average eGFR value was 41 (19.5 – 89) ml/min per 1.73 m^2 .

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The copy numbers (median) of miR-148b in IgAN and healthy controls were 11742 (5224 – 17693) and 4032 (2970 – 5342), respectively. The copy numbers (median) of let-7b in IgAN and healthy controls were 1124 (202 – 2809) and 205 (167 – 435), respectively. The levels of miR-148b and let-7b in IgAN were 2.91 and 5.48 times higher than the healthy controls and were statistically significantly different with p values 0.0002 and 0.005, respectively.

Based on spearman's rank correlation analysis, where the correlation coefficient was more than +/- 0.33 and p value was less than 0.05, we found miR-148b in negative correlation with S, T, systolic and diastolic blood pressure, and positively correlated with eGFR (Table 1).

Table 1

Correlation of microRNA with predictive markers of IgA nephropathy

microRNA		Predictive marker	ρ	p
miR-148b	~	S	- 0.4	0.028
	~	T	- 0.54	0.002
	~	Systolic BP	- 0.49	0.006
	~	Diastolic BP	- 0.42	0.019
	~	eGFR	0.533	0.002
let-7b	~	C3	0.36	0.047

ρ: Correlation coefficient (Spearman's rank correlation); p: probability; S: Segmental glomerulosclerosis; T: Tubular atrophy/Interstitial fibrosis; BP: Blood pressure; eGFR: Estimated glomerular filtration rate; C3: Complement

The miR-148b level was found higher in M1 and E1 than M0 and E0, respectively. There was no statistically significant difference found in miR-148b level in different stages of M and E. Furthermore, miR-148b was found decreased with the increase of S, T, C, and CKD stages (Figure 1). miR-148 was found statistically differentiating the S0 and S1, T0 and T1, C0 and C2, and CKD3 and CKD4 stages with p values of 0.03, 0.003, 0.033, and 0.013, respectively.

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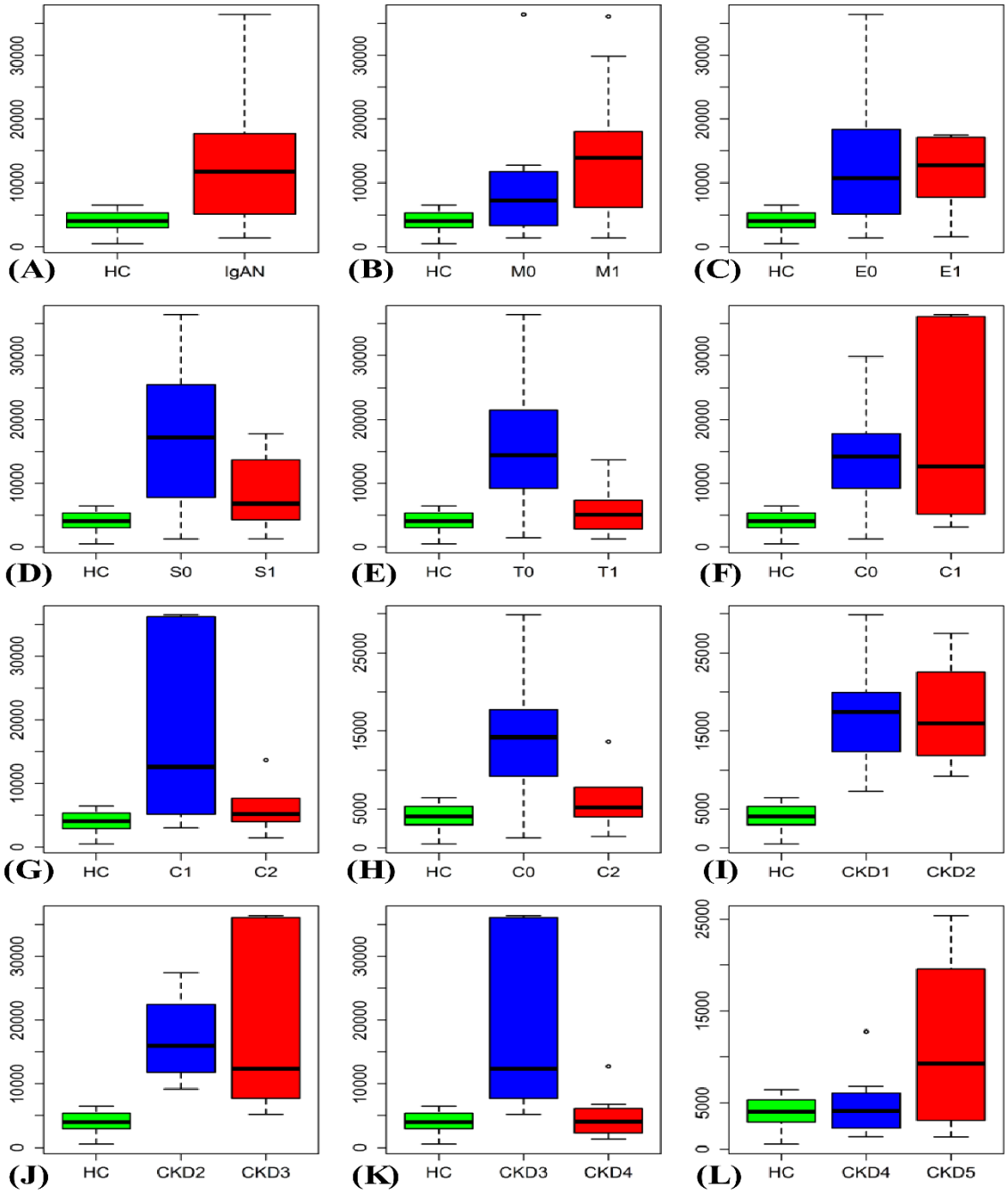

Figure1: Variation in miR-148b quantity along with IgA nephropathy disease conditions and in healthy controls

HC: Healthy control, IgAN: IgA nephropathy; Y axis shows microRNA concentration that is expressed in copy number/µl RNA(isolated RNA eluted in 20 µl nuclease free water; M0, M1, E0, E1, S0, S1, T0, T1, C0, C1, and, C2 are kidney biopsy interpretation score of MEST-C oxford scoring system for the IgAN; CKD1: Chronic kidney disease stage 1; CKD2: Chronic kidney disease stage 2; CKD3: Chronic kidney disease stage 3; CKD4: Chronic kidney disease stage 4; CKD5: Chronic kidney disease stage 5

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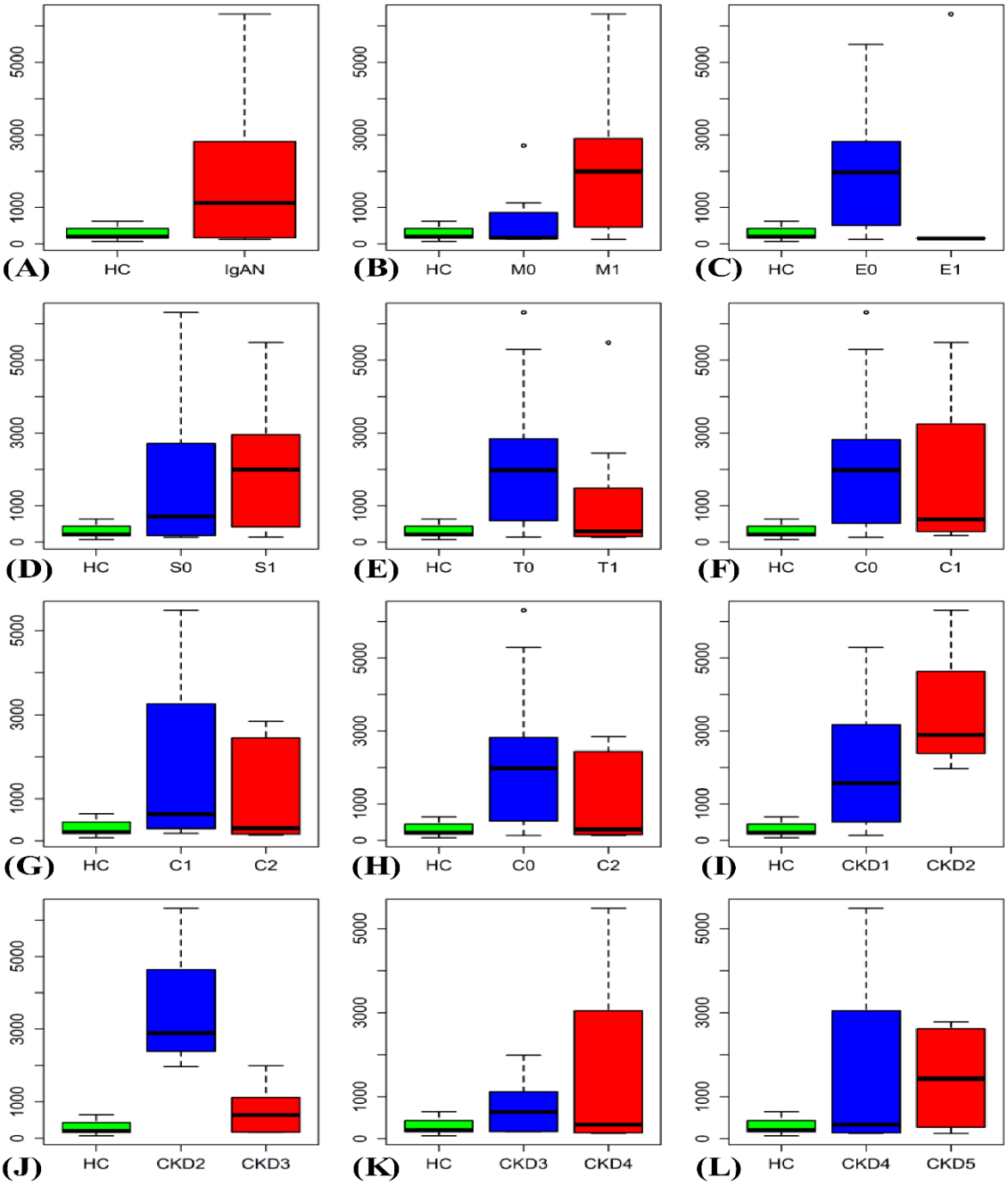

Figure2: Variation in let-7b quantity along with IgA nephropathy disease conditions and in healthy controls

HC: Healthy control, IgAN: IgA nephropathy; Y axis shows microRNA concentration that is expressed in copy number/µl RNA(isolated RNA eluted in 20 µl nuclease free water; M0, M1, E0, E1, S0, S1, T0, T1, C0, C1, and, C2 are kidney biopsy interpretation score of MEST-C oxford scoring system for the IgAN; CKD1: Chronic kidney disease stage 1; CKD2: Chronic kidney disease stage 2; CKD3: Chronic kidney disease stage 3; CKD4: Chronic kidney disease stage 4; CKD5: Chronic kidney disease stage 5

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The let-7b level was found increased in M1 and S1 than MO and S0, respectively. Furthermore, the level of let-7b was found decreased with an increase in S, T, and C scores (Figure 2). The CKD stage saw a zigzag in let-7b level. There was a statistically significant different found in let-7b level between CKD stage 2 and 3 with p value 0.02. We could not find let-7b statistically differentiating any other predictive stages of IgAN.

Based on logistic regression models, miR-148b was found independently differentiating the tubular atrophy/interstitial fibrosis and crescents state in glomeruli. The sensitivity, specificity, and AUC of ROC for miR-148b against T were found at 0.87, 0.77, and 0.85, respectively. The same way, the sensitivity, specificity, and AUC of ROC for crescent stage, C0, and C2 were found at 0.83, 0.77, and 0.79, respectively. The threshold value of mir-148b was found the same in both cases at 8479 copy numbers. The miR-148b was also showed good predictive value to differentiate the CKD stage 3 and 4 with sensitivity, specificity, and AUC of ROC at).87, 0.83, and 0.89, respectively. The threshold value of miR-148b was found a bit lower at 7266 for CKD stage. We could not find let-7b as a good predictive marker for IgAN.

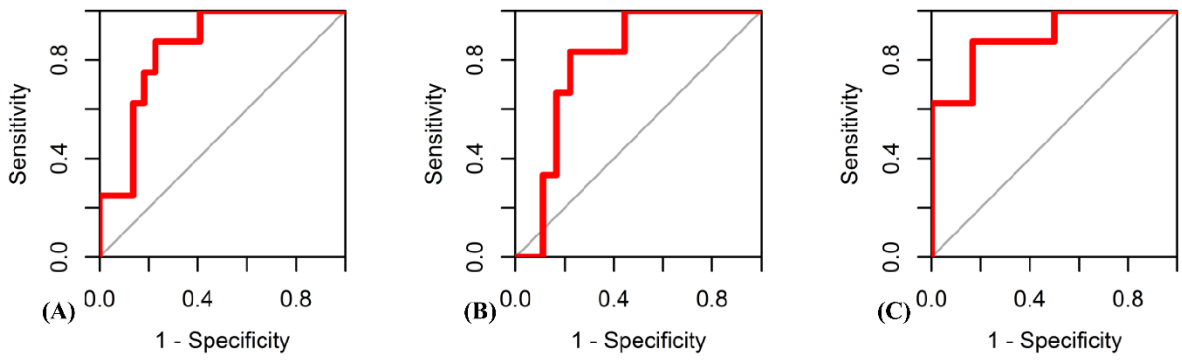

Figure3: Predictive properties of microRNA in IgA nephropathy
(A): Receiver operating characteristic (ROC) curve of miR-148b with respect to T (Tubular atrophy/Interstitial fibrosis); (B): ROC of miR-148 with respect to C (Crescents); (C): ROC of miR-148 with respect to chronic kidney disease (CKD) stages

 doi:10.20944/preprints202006.0049.v1

Discussion

G Serino had reported that let-7b was upregulated and mir-148b downregulated in serum samples of IgAN than healthy controls[17]. They proposed let-7b together with miR-148b as markers for IgAN. In our study, miR-148b and let-7b both were upregulated by 2.91 and 5.49 times, respectively, than the healthy controls. If we compare our result with the previous study, which was a multicenter study spread over Italy, Greece, Japan, and China, we found a contradictory result. miR-148b level in plasma was almost three times higher than healthy participants. The level of let-7b was also more than five times higher than the healthy controls, while in the previous study it was around two to three times higher than the HC. There are differences between our study and the previous multicenter study. They worked on the serum sample while we used plasma sample and another major reason was the different population. We do not think that serum or plasma would make a big impact on the result of miRNA level. In this case, different demography certainly could have made a difference as ours was a single center study.

Furthermore, we studied the prognostic value of miR-148b and let-7b. Though let-7b was more than five times higher in IgAN participants than the HC, still it could not differentiate any of the kidney biopsy histopathological stages. While, miR-148b was able to differentiate the S and T stages irrespective of its lower level increase in IgAN than HC in comparison to let-7b. Tubular atrophy/interstitial fibrosis is considered as an independent parameter to prognose the disease severity[11]. At more than 80% sensitivity and AUC, miR-148b alone was able to distinguish the different stages of T. miR-148b was negatively correlated to S, T, and BP and positively to eGFR. We found an unusual behave between miR-148b and IgAN severity. In the preliminary stage of IgAN, miR-148b level was found higher in IgAN, but with the increase in disease severity, miR-

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148b level was decreased. The average copy number of miR-148b in T1 stage was 14422 and in T0 was 5072. The average copy number of miR-148b in healthy controls, was found 4032 that lied between the inter quartile range (IQR) of miR-148b in T0. The same way, the copy numbers of miR-148b in C0, C1, and C2 were 14191, 12602, and 5202, respectively, where the lower IQR of C2, 4222 was close to the average copy number of miR-148b in healthy controls. Furthermore, the average miR-148b copy number (4112) in CKD stage four was almost same to the average copy number (4032) of miR-148b in healthy controls. It means that miR-148b cannot differentiate the severe form of IgAN and healthy controls, but it can very well differentiate the preliminary stage of IgAN with healthy controls. Furthermore, the threshold of miR-148b copy number to differentiate the T0 and T1 and C0 and C2 stages was the same at 8479. The threshold of miR-148b to differentiate CKD stages three and four was found at 7266. Therefore, we can fix a cutoff point for miR-148b to differentiate the disease severity. 8479 could be used as a disease severity mark for IgAN, where 7266 could be used as another deteriorating condition. Therefore, once IgAN is confirmed, then decreased miR-148 could be used as a predictive tool for the progression of the disease.

This study validates miR-148b as a prognostic biomarker. Repeat biopsy is not done frequently, so miR-148b could be used in place of repeat biopsy as a prognostic biomarker. It can also be considered as a prospective diagnostic tool for the detection of IgAN among patients with reported kidney dysfunction at an early stage.

Conflict of interest: The authors declare that they have no conflict of interest.

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