ORIGINAL ARTICLE

Elucidation of mRNA targets of *miR-145-5p* in diabetic kidney disease using bioinformatics analysis

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ABSTRACT

Introduction: Diabetic kidney disease (DKD) is a major global cause of end-stage-kidney disease. In view of its on-going risk of disease progression, the search for a better biomarkers and treatment led to the discovery of microRNAs which regulate gene expression post-translationally. Recently, we reported a trend of upregulation of *miR-145-5p* in sera of type 2 diabetic patients with macroalbuminuria in a selected Malaysian population, which concurred with previous *in vivo* and *in vitro* studies of DKD. In addition, miR-145 has been implicated as a tumour suppressor in various cancers. **Methods:** In this study, bioinformatics tools were utilized to predict the mRNA targets of *miR-145-5p*. **Results:** A total of 683 and 224 experimentally-validated mRNA targets of *miR-145-5p* were identified by Tarbase and miRTarbase, respectively. Eighty-six (86) commonly identified targets were submitted to Metascape and Enrichr for enrichment analysis. Bioinformatics analysis and literature search suggested that insulin receptor substrate 1 (IRS1) was the most promising target of *miR-145-5p*. Its associated Gene Ontology terms and pathways included insulin-like growth factor receptor signalling and Forkhead transcription factors (FOXO), respectively. Based on these analyses, the roles of IRS1 in DKD were proposed. **Conclusion:** As the kidneys are heterogenous in cell types and the mechanism of miRNA is cell-type-dependent, target prediction of *miR-145-5p* by bioinformatics analysis is particularly important in DKD, to improve the likelihood of a successful *in vitro* experimental verification in specific renal cell types. In addition, this study attempts to utilize bioinformatics studies, which is not widely done in DKD, as recently reported.
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Keywords: Diabetic kidney disease, *miR-145-5p*, mRNA targets, Bioinformatics, *in silico*

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INTRODUCTION

Currently, diabetic kidney disease (DKD) is a global burden as the leading cause of chronic kidney disease (CKD) (1). Consequently, DKD continues to be the major cause of end-stage-kidney disease (ESRD) worldwide, accounting for 69% (2) and 39% (3) of cases in Malaysia and the U.S.A, respectively. Despite current biomarkers and newer therapeutic agents, the risk of disease progression remains (1). Thus, there is an ongoing need to further understand the underlying mechanisms of DKD in search for a better diagnostic or therapeutic agent (1).

The pathogenesis of DKD begins in the glomeruli, whereby hyperglycaemia induces activation of intracellular signalling pathways which ultimately culminates in renal fibrosis (1). The pathological paradigm has since expanded beyond glomeruli and recognized the roles of new factors (4). One of the emerging factors is epigenetic modification, which has been implicated in the early stages of DKD, its self-perpetuation and the phenomenon of metabolic memory (5). Epigenetic modification may partially explain familial and ethnic clustering of DKD (4). One of epigenetic modifications is a family of small non-coding RNAs, known as microRNAs (4).

miRNA regulates post-translational gene expression by binding to a seed sequence in the 3’ UTR of a mRNA target with complementary sequence to its 5’ end (6). Alteration in gene expression is brought about by either a perfect or partial complementarity, thereby causing mRNA destabilization or translational repression, respectively, or a combination of both (7). Partial

complementarity also allows each miRNA to target several mRNAs and in turn, each mRNA can be targets of several miRNAs (7). This suggests a complex network between miRNAs and their mRNA target, identification of which is key step in unraveling the regulatory roles of miRNAs in gene expression (8).

Previous studies have shown dysregulation of miRNAs in the pathogenesis of DKD (9). Our recent *in vivo* findings concluded a trend of upregulation of *miR-145-5p* in T2DM patients with macroalbuminuria compared to those with normoalbuminuria as it did not reach statistical significance (10). Interestingly, a similarly statistically non-significant upregulation of *miR-145* has been previously reported in sera of patients with CKD, including some DKD patients (11). As cited in our previous study (10), an upregulation of *miR-145* has been reported in plasma (12) and urine (13-14) of type 1 DM patients with DKD, as well as in urine of patients with CKD (15). Similarly, upregulation of *miR-145-5p* has been reported in animal models of DKD (13). *In vitro miR-145-5p* overexpression in response to high glucose has been reported in human mesangial cells (13). In contrast, *miR-145-5p* overexpression inhibited high glucose-induced apoptosis in podocyte (16); whilst in colon cancer cells, *miR-145* has been implicated as a tumour suppressor (17).

In this study, we have utilised microRNA target prediction and enrichment analysis bioinformatics tools in combination with in-depth literature review to select the most likely functional mRNA targets of *miR-145-5p*. Based on these analyses, their molecular mechanisms in DKD were subsequently proposed.

Structurally, the kidneys comprise of glomerular, tubular and interstitial functional compartments, with each compartment in turn being made up of various types of renal cells (18). In view of such complexity and heterogeneity of the kidneys, and the variability of miRNAs effects in different cells (19), bioinformatics analysis, particularly in DKD, may contribute towards a successful *in vitro* experimental verification. In addition, lack of bioinformatics studies in DKD has been recently reported (20), a gap which this study attempts to contribute to.

MATERIALS AND METHODS

Expression of *miR-145-5p*

The upregulated pattern of *miR-145-5p* was concluded from our previous study (10), in which, the differential expression of a group of miRNAs involved in human fibrosis was done by a profiler RT-qPCR using sera of type 2 diabetic patients with and without albuminuria from a selected Malaysian population. The study obtained an ethical approval from the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia [NMRR-17-2715-38571 (IIR)] (10). In that study, inclusion criteria were T2DM patients and available clinical and laboratory information; whilst exclusion criteria were patients with type 1 DM, non-diabetic kidney diseases, comorbidities such as cancer, acute febrile illness or recent infections, pregnancy and postpartum (10). Blood was collected from a total of 41 patients and RNA was extracted using miRNeasy Serum/Plasma Kit (Qiagen GmbH, Hilden, Germany) (10). RT-qPCR analysis was done in 12 and 33 sera using Pathway-Focused (Human Fibrosis) miScript miRNA qPCR Array (Qiagen GmbH, Hilden, Germany) and a custom miScript miRNA qPCR array (CMIHS02742) (Qiagen GmbH, Hilden, Germany), in the discovery and validation cohort, respectively (10). Relative expression (RE) was derived by the $2^{-\Delta Ct}$ method whereby expression of individual miRNA was normalized to that of the housekeeping gene SNORD95 and fold change was from the normalized miRNA expression in each test group (micro- or macroalbuminuria) divided by the normalized miRNA expression in the control group (normoalbuminuria) (10). The p values were calculated based on an independent samples t-test of the replicate RE ($2^{-\Delta Ct}$) values for each gene in the control and test groups (10).

Identification of mRNA targets of *miR-145-5p*

mRNA targets of *miR-145-5p* were identified from two experimentally validated miRNA-target gene interactions databases, TarBase v8 (21) and miRTarBase release 8.0 beta (22). mRNA targets commonly identified by these two databases were chosen for further analysis. TarBase uses manually curated interactions in compiling their database (21), whilst MirTarbase, uses text mining techniques and manual curation (22). In these databases, the putative target genes are ranked based on the strength of experimental techniques used to validate miRNA:target interaction. The direct low-throughput techniques such as the reporter assay ranks the highest, followed by indirect low-throughput methods, such as RT-qPCR, direct high-throughput methods such as CLIP chimeric method and finally, indirect high-throughput methods such as CLIP-sequencing (21). In addition, a higher score means an article is more related to a miRNA-target interaction (22).

Selection of the most likely mRNA target of *miR-145-5p*

The most promising mRNA target(s) of *miR-145-5p* was selected based on the principle of reciprocal expression, whereby a high probability miRNA-target pair is likely when an upregulation of miRNA is accompanied by a downregulation of its mRNA target (23). Accordingly, to identify the most promising mRNA targets of *miR-145-5p*, a comprehensive review of the literature published up to 2021 were performed between March 1 2021 and September 15 2021, on electronic databases, including Google Scholar, Scopus and PubMed using the following keywords: 'diabetic kidney', 'diabetic nephropathy', 'chronic kidney disease', '*miR-145-5p*' and 30% of top-

ranked putative mRNA targets of *miR-145-5p*.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of mRNA targets of *miR-145-5p*

The commonly identified experimentally-validated mRNA targets of *miR-145-5p* found in both Tarbase and MirTarBase were submitted to Enrichr (24) and Metascape (25). In Enrichr, the input genes are compared to about 300 gene set libraries and the output of enrichment terms are ranked based on p-value derived by Fisher's exact test or the hypergeometric test (24). Metascape uses hypergeometric test and Benjamini-Hochberg p-value correction algorithm, integrating annotation information from over 40 knowledgebases, including Gene Ontology, KEGG and Reactome (25). Its bar graph displays top non-redundant enrichment clusters, ranked according to statistical significance, defined as $p<0.05$ (25).

RESULTS

Identification of the mRNA targets of *miR-145-5p*

A total of 683 and 224 mRNA targets of *miR-145-5p* were found in Tarbase and miRTarbase, respectively. Eighty-six (86) commonly identified experimentally-validated targets of *miR-145-5p* were submitted for enrichment analysis.

Selection of the most likely mRNA target of *miR-145-5p*

Following a thorough literature search, the most promising mRNA target(s) of *miR-145-5p* (out 86 identified targets) was/were chosen based on the principle of reciprocal expression, whereby when a miRNA is upregulated, its mRNA target will be downregulated (23). Insulin receptor substrate 1 (IRS1) was shown to be the most promising putative mRNA target of *miR-145-5p*, followed by insulin receptor substrate 2 (IRS2) and SRY-box 2 (SOX2), as indicated by their consistent reciprocal dysregulation in response to upregulated *miR-145-5p*, as revealed by our literature review.

Gene Ontology (GO) enrichment analysis of mRNA targets of *miR-145-5p*

Fig. 1 shows Gene Ontology (GO) analysis by Enrichr for the 86 putative targets of *miR-145-5p*. For these 86 putative mRNA targets of *miR-145-5p*, GO analysis revealed that the most enriched GO BP terms included negative regulation of cell adhesion and wound healing, spreading of epidermal cells (Fig. 1A); whilst the most enriched GO MF terms included cadherin binding and insulin receptor binding (Fig. 1B); and the most enriched GO CC terms included nuclear chromatin and chromatin (Fig. 1C). GO analysis of IRS1 showed that its most enriched GO BP terms included insulin-like growth factor receptor signalling, regulation of fatty acid oxidation and glucose metabolic process; whilst its most enriched GO MF terms included insulin

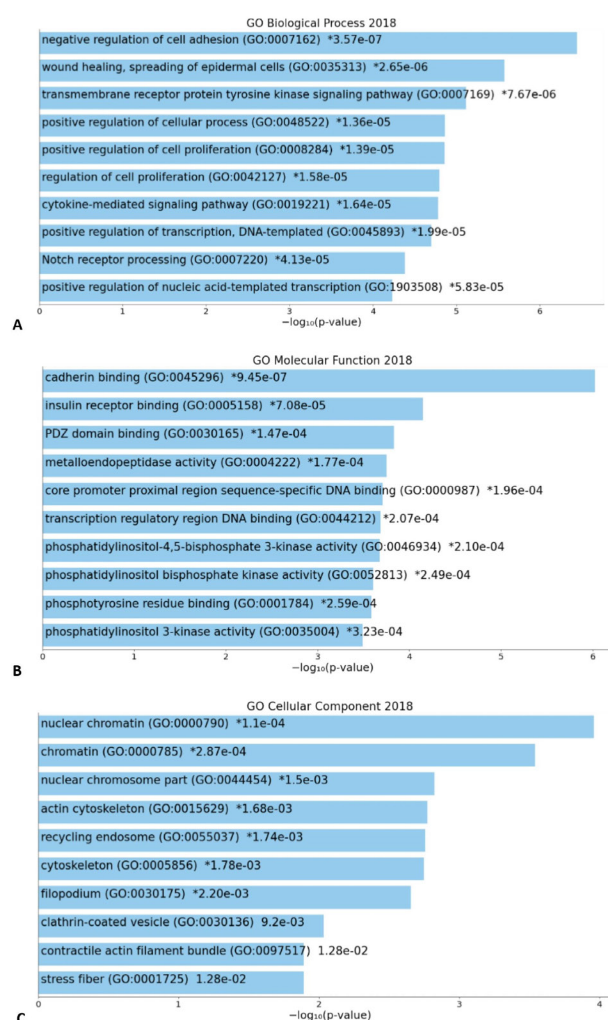

Figure 1: Gene Ontology (GO) analysis of the putative mRNA targets of *miR-145-5p* (a) Biological Processes; (b) Molecular Functions; (c) Cellular Components

receptor binding, phosphotyrosine residue binding and phosphatidylinositol-4,5-bisphosphate 3 kinase activity (Fig.1B). Meanwhile, the most enriched GO CC term associated with IRS1 was protein kinase complex.

Pathway functional enrichment analysis of mRNA targets of *miR-145-5p*

Fig. 2 shows the most enriched KEGG pathways, by Enrichr, and Fig. 3 shows the most enriched terms, by Metascape, of the 86 putative mRNA targets of *miR-145-5p*. For these putative mRNA targets of *miR-145-5p*, the most enriched KEGG pathways included proteoglycans in cancer, bladder cancer and breast cancer (Fig.2); and their most enriched terms included diseases of signal transduction by growth factor receptors and second messengers, regulation of cell adhesion and Vascular Endothelial Growth Factor-A-Vascular Endothelial Growth Factor-Receptor 2 (VEGFA-VEGFR2) signaling pathway (Fig. 3). In this study, further analysis revealed that the most enriched KEGG pathways associated with IRS1 included Forkhead transcription factors (FoXO) (Fig.2), AMP-activated protein kinase (AMPK), phosphatidylinositol 3 kinase/protein kinase B (P13k-

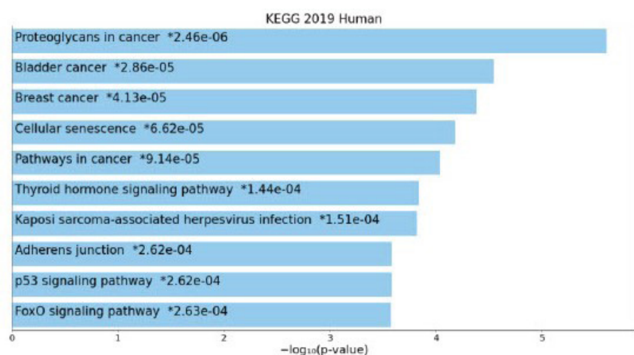

Figure 2: The most enriched KEGG pathways of the putative mRNA targets of *miR-145-5p*

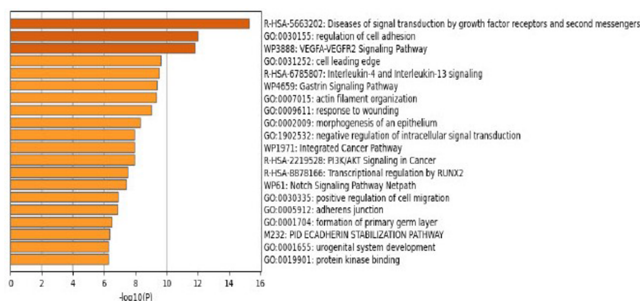

Figure 3: The most enriched terms of the putative mRNA targets of *miR-145-5p*

Akt) (Fig. 3) and mammalian target of rapamycin (mToR) signalling pathways.

DISCUSSION

In this study, as sequence-based miRNA target prediction tools tend to have a high rate of false positive (8), we have chosen experimentally-validated databases to identify the putative mRNA targets of *miR-145-5p*. In this study, the putative mRNA targets commonly identified by both databases were selected, as targets predicted by more than one database are more likely to be true than other targets, as previously highlighted (26). The practice of selecting mRNA targets which were predicted by at least 2 of 3 databases had been previously applied (27), whereby targets which were consistently obtained using both databases were considered the likely mRNA targets (28).

Functional pathway analysis in this study highlighted several distinct pathways of relevance to DKD pathophysiology, such as adherens junction, p53 and FoxO signalling pathways and other pathways as potential targets of *miR-145-5p* (Fig.2), involving the putative targets. Literature search revealed that insulin receptor substrate 1 (IRS1) was probably the most likely putative target of *miR-145-5p* and will be discussed further. The transcript length of IRS1 gene is 9,771 bases (GenBank Accession: NM_005544) (29). The *miR-145-5p* binds to the 3'-UTR (untranslated region) of IRS1 mRNA at two putative positions 2473-2480 and 2337-2343, based on prediction by the online bioinformatics tool, TargetScan (30). Shi et al., 2007 (31) had demonstrated that the two (2) binding sites of miR-145 on the 3'UTR of the mature mRNA IRS1 started at nucleotide (nt) 1 and nt 173 of the 3'UTR of the IRS1 gene.

Failure of insulin actions, either due to lack of insulin or insulin resistance in T1DM and T2DM, respectively is pathognomonic of diabetes mellitus. Earlier evidence implicating insulin resistance has been observed in *in vitro* model of insulin resistance in podocyte (32). A concept of selective insulin resistance was initially supported by evidence showing impaired IRS1 signalling pathway in *ex vivo* rat glomeruli while sparing the tubules (33), thus supporting the notion of the former being the initial target of dysfunction in DKD (34). Using an *ex vivo* rat glomerular mesangial cells (GMCs), it was showed that not only hyperglycaemia in the glomeruli inhibited selective insulin downstream signalling pathways, but it was also accompanied by selective loss of IRS1 and not IRS2, via an increased ubiquitin-associated degradation (33). Subsequently, selective insulin resistance mediated by similar reciprocal loss of IRS1 but preserved IRS2 function was also reported in renal tubules (35).

It was reported presence of insulin receptor and IRS1 across all the glomerular cell types, the highest being in podocytes, followed by mesangial and endothelial cells (33). In particular, the presence of insulin signalling pathway in mesangial cells was in keeping with a previous study which reported significantly reduced insulin-stimulated phosphorylation of IRS1 and Akt kinase was seen following expression of IRS1 mutant Arg972 in human mesangial cells (36). In contrast to Mima et al. (2011) (33), Santamaria et al. (2015) (37) however showed absence of IRS1 expression in GPC, instead, insulin resistance seen in these cells was attributed to reduced expression of IRS2.

Previous evidence showed that *miR-145* has a role as a tumour suppressor (17), by downregulating its target IRS-1 (17, 37), cellular proliferation is thus inhibited in favour of differentiation. Furthermore, miR-145 has been shown to be specifically expressed in GMCs (38). The findings pertaining to mesangial cells are particularly encouraging. These include the *in vitro* findings of hyperglycaemia-induced *miR-145* upregulation (13) and IRS1 expression (33, 36) in human mesangial cells. Subsequent selective inhibition of IRS1 and its insulin-related downstream signalling pathway occurring post-translationally is of particular interest for us to explore. Previous studies showed that inhibition of mesangial cell apoptosis which led to enhanced mesangial cell proliferation by insulin occurred via activation of PI3K pathway, as mentioned in Horita et al. (2016) (35); in keeping with known function of insulin as a pro-mitogenic, anti-apoptotic and anti-differentiation factor (39).

In this study, several of the most enriched GO CC terms were in line with IRS1 cellular position and functions,

placing it within cytosol and plasma membrane and in contact with intracellular membrane-bound organelle (Fig.1A). Similarly, the most enriched GO BP terms associated with IRS1 included insulin-like growth factor receptor signalling signal transduction. The most enriched KEGG pathways of IRS1 included FoxO, AMPK, P13k-Akt and mTOR signalling pathways (Fig. 2 and Fig. 3).

IRS1 and IRS2 are members of IRS family of docking proteins which transmit signals from insulin-receptor and insulin-like growth factor-1 to intracellular pathways (39). IRS primarily activates phosphoinositide 3-kinase (PI3K)–protein kinase B (PKB/Akt) cascade which is involved in regulation of many downstream effectors such as glycogen synthase kinase 3β (GSK3β), mTOR and FoxO (40). IRS1 exert its pro-mitogenic, anti-apoptotic effect probably via Akt which has been shown to increase BCLxL anti-apoptotic protein via its inhibitory effect on pro-apoptotic protein Bad, inhibit cell-death protease caspase-9, reduce transcription of death-activating Fas ligand via its inhibitory effect on FOXO and increase expression of anti-apoptotic genes via its stimulatory effect on nuclear factor-kB (NF-kB) (41-43).

Taken together, we postulate that upregulation of *miR-145-5p* is the probably the unidentified upstream player previously suggested (34), which upon induction by hyperglycaemia, leads to reduced translation of IRS1 with resultant increased apoptosis in mesangial cells. Such miRNA-dependent, post-translational non-ubiquitin-associated degradation is not foreign and has been shown for other mRNA targets previously mentioned (44). Opposite modulation independently observed in podocytes, whereby downregulation of miR-145 (16) and reduced IRS2 expression (35), reflecting varying miRNA modulation in different cell types (19), further point us towards verifying our findings in GMC in the future *in vitro* experiment. Furthermore, GMC apoptosis, induced by hyperglycaemia has been observed in DKD (45, 46).

Thus, our proposed role of *miR-145-5p* and its target IRS1 in mesangial cells is as depicted in Fig.4. Furthermore, the significant correlation of *miR-145-5p* with eGFR (p=0.05) as shown in our recent *in vivo* human study (10) further indicate that upregulation of miR-145-5 may contribute towards the pathogenesis of DKD, which is also in keeping with previous findings that carriers of mutant IRS1 gene having significantly lower GFR (36). Interestingly, whilst both IRS1- and IRS-2 knockout mice displayed microalbuminuria (47), carriers of mutant IRS1 have also been associated with increased microalbuminuria (48), implicating its involvement (47).

In this study, the most likely mRNA target of *miR-145-5p* identified from 2 experimentally-validated miRNA-target databases was IRS1. Its most enriched gene ontology

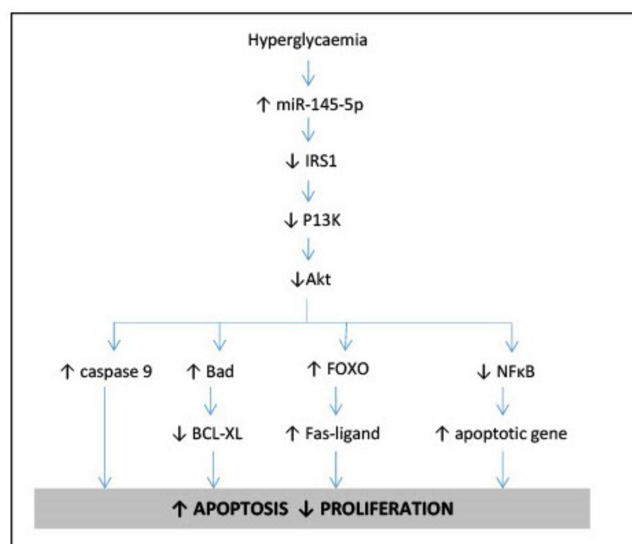

Figure 4: Proposed roles of miR-145-5p in DKD. P13K: phosphoinositide 3-kinase; FOXO: Forkhead transcription factors; NF-kB: nuclear factor-kB

terms and pathways included insulin-like growth factor receptor signalling and P13k-Akt. Our bioinformatics and bibliographical analyses indicate that *miR-145-5p* may act as a negative regulator in the progression of DKD through involvement of its putative target, IRS1, in insulin signalling pathway.

To verify our prediction, some of the suggested *in vitro* experiments include the following. Firstly, verification of *miR-145-5p*: IRS1 as a miRNA-target pair needs to be carried out, ideally by luciferase 3'UTR reporter assay. Secondly, an overexpression of *miR-145-5p* in a specific renal cell in response to diabetic stressors such as hyperglycaemia or oxidative damage will need to be verified. Previous findings on miR-145 (13, 38) and IRS1 (33, 36) indicated that *in vitro* verification should be done in GMCs. Subsequently, the proposed downregulation of IRS1 in response to an upregulated *miR-145-5p* in these cells needs to be verified by either measuring the expression level of IRS1 mRNA by RT-PCR or that of its target protein by western blotting (49). Verification of predicted phenotypic and functional changes and reversal of these changes may be achieved by means of *miR-145-5p* knock-in and knock-out, respectively (50). Based on previous studies, an upregulation of *miR-145-5p* was proposed to lead to increased apoptosis in GMCs which can be verified by an apoptosis ELISA assay (42) or by flow cytometry (43); whilst the proposed reduced cellular proliferation may be assessed by cell viability assay (43). Western Blot analysis may be used to verify the proposed increased of the downstream proteins including PI3K, Akt, caspase 9, Bad and NFκβ (42, 43). Alternatively, a Caspase 9 assay may be used to measure its activity (42).

CONCLUSION

In conclusion, bioinformatics and bibliographical

analyses in this study may improve the probability of a successful *in vitro* verification experiments. This is particularly important in an organ with complex structure as kidneys (18). Identification of the most likely renal cell type is also crucial as it has been previously suggested that miRNA modulation is cellular-specific (19). Furthermore, this study attempts at utilization of bioinformatics analysis is timely in view of its recently reported scarcity in DKD (20).

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