ISSN 2220-6124 (online)

World Journal of *Nephrology*

World J Nephrol 2018 March 6; 7(2): 51-70

Published by Baishideng Publishing Group Inc

World Journal of Nephrology

Contents

Bimonthly Volume 7 Number 2 March 6, 2018

NAME OF JOURNAL
World Journal of Nephrology

ISSN
ISSN 2220-6124 (online)

LAUNCH DATE
February 6, 2012

FREQUENCY
Bimonthly

EDITORIAL OFFICE
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World Journal of Nephrology
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7901 Stoneridge Drive,
Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
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Suite 501, Pleasanton, CA 94588, USA
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PUBLICATION DATE
March 6, 2018

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World Journal of Nephrology

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World J Nephrol 2018 March 6; 7(2): 51-57

DOI: 10.5527/wjn.v7.i2.51

ISSN 2220-6124 (online)

REVIEW

Is serum copeptin a modifiable biomarker in autosomal dominant polycystic kidney disease?

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Author contributions: Tasneem M contributed to draft the manuscript, review the literature and the content; Mannix C contributed to draft the manuscript and contributed to the content; Wong A contributed to draft the manuscript and the content; Zhang J contributed to draft the manuscript and contributed to the content; Rangan G contributed to draft the manuscript, review the literature and contributed to the content.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported. Rangan G is recipient of an investigator initiated grant from the Danone Nutricia Research (manufacturer of bottled water).

Manuscript source: Unsolicited manuscript

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Received: December 18, 2017
Peer-review started: December 18, 2017
First decision: January 23, 2018
Revised: January 29, 2018
Accepted: February 28, 2018
Article in press: February 28, 2018
Published online: March 6, 2018

Abstract

The availability of disease-modifying drugs for the management of autosomal dominant polycystic kidney disease (ADPKD) has accelerated the need to accurately predict renal prognosis and/or treatment response in this condition. Arginine vasopressin (AVP) is a critical determinant of postnatal kidney cyst growth in ADPKD. Copeptin (the C-terminal glycoprotein of the precursor AVP peptide) is an accurate surrogate marker of AVP release that is stable and easily measured by immunoassay. Cohort studies show that serum copeptin is correlated with disease severity in ADPKD, and predicts future renal events [decline in renal function and increase in total kidney volume (TKV)]. However, serum copeptin is strongly correlated with creatinine, and its additional value as a prognostic biomarker over estimated glomerular filtration rate and TKV is not certain. It has also been suggested that copeptin could be a predictive biomarker to select ADPKD patients who are most likely to benefit from AVP-modifying therapies, but prospective data to validate this assumption are required. In this regard, long-term randomised clinical trials evaluating the effect of prescribed water intake on renal cyst growth may contribute to addressing this hypothesis. In conclusion, although serum copeptin is aligned with the basic pathogenesis of ADPKD, further rigorous studies are needed to define if it will contribute to enabling the delivery of personalised care in ADPKD.

Key words: Polycystic kidney disease; Copeptin; Biomarker

Core tip: Serum copeptin is correlated with disease severity in autosomal dominant polycystic kidney disease (ADPKD), and predicts future renal events (decline in renal function and increase in total kidney volume). The aim of this review is to critically evaluate the role of copeptin as a prognostic biomarker of renal outcomes in ADPKD, and if it has potential as a predictive marker of treatment response.

Tasneem M, Mannix C, Wong A, Zhang J, Rangan G. Is serum copeptin a modifiable biomarker in autosomal dominant polycystic kidney disease? *World J Nephrol* 2018; 7(2): 51-57 Available from: URL: http://www.wjgnet.com/2220-6124/full/v7/i2/51.htm DOI: http://dx.doi.org/10.5527/wjn.v7.i2.51

INTRODUCTION

The life-time risk for end stage kidney disease (ESKD) in autosomal dominant polycystic kidney disease (ADPKD) is characterised by high intra- and interfamilial variability[1]. Epidemiological data indicates that only 50% of patients with ADPKD will develop ESKD by the age of 60[2]. This variability in risk for ESKD is likely due to the interaction of genic factors[3] with environmental variables[4] that alter the expressivity of the clinical phenotype[5,6]. Routine tests performed during the initial clinical evaluation of affected patients, such as estimated glomerular filtration rate (eGFR), lack sensitivity as prognostic markers in early disease[7], and other clinical information (such as family history of early-onset of ESKD) do not have precision[8]. The uncertainty in predicting renal prognosis causes tremendous anxiety to patients and their families[9]. Furthermore, the introduction of disease-modifying drugs to treat ADPKD has catalysed an urgent need to identify and validate a panel of reliable and easily measurable clinical, genetic, molecular and imaging biomarkers in predicting the risk for ESKD[10].

The arginine vasopressin (AVP)-cAMP signalling pathway has a central role in the initiation of lifetime growth of kidney cysts in ADPKD[11]. Serum copeptin is a surrogate marker of AVP release[12], and the availability of a sensitive commercially available immunoassay, has led to several cohort studies to evaluate its role as a prognostic biomarker in ADPKD. The role of copeptin as a clinical diagnostic test is also under evaluation in several other diseases including in the differential diagnosis of polyuria-polydipsia (where it is being considered as a replacement for the direct measurement of serum AVP)[13,14], and in the diagnostic evaluation of other disorders (such as acute myocardial infarction[15] and sepsis[16]) where its potential utility is less certain. In this regard, to date, copeptin is not funded for reimbursement for routine measurement in patients in ADPKD in any country, providing an indirect indicator that its value as a prognostic biomarker in this setting has also not been proven and that further data is needed. The aim of this review is to critically evaluate the role of copeptin as a prognostic biomarker of renal outcomes in ADPKD, and if it has potential as a predictive marker of treatment response.

ROLE OF AVP-CAMP SIGNALLING IN THE PATHOGENESIS OF RENAL CYST GROWTH IN ADPKD

The role of AVP in the pathogenesis of ADPKD has been reviewed elsewhere[11], but briefly, it is considered to be the most important factor that determines the postnatal rate of renal cyst growth. The most compelling preclinical evidence to support this hypothesis is that the congenital deficiency of AVP almost completely abrogated the formation of renal cysts in the pck rat[17]. Furthermore, in mouse models of PKD, small-molecule vasopressin-receptor antagonists were highly effective in reducing kidney cyst growth[18]. In humans, the evidence is supported by the TEMPO 3:4 and REPRISE trials which collectively showed that tolvaptan (a highly specific vasopressin-receptor antagonist) reduced the rate of increase in total kidney volume (TKV) in early-stage ADPKD and also the decline in renal function in late-stage disease[19,20]. Given its critical importance, it seems logical to consider markers of AVP release as potential biomarkers in ADPKD.

SYNTHESIS, FUNCTION AND DEGRADATION OF COPEPTIN

Synthesis of copeptin

As shown in Figure 1, copeptin is the C-terminal end of the AVP precursor molecule (pre-proAVP), a 164-amino acid peptide consisting of four segments: (1) The signal peptide at the N-terminus (amino acids 1-19); (2) AVP (amino acids 20-28); (3) neurophysin (amino acids 32-124); and (4) copeptin (amino acids 126-164), a 39-aminoacid glycopeptide that makes up the C-terminal part of pro-AVP. As summarised in Table 1, the precursor peptide (pre-proAVP) is produced in two anatomically distinct regions of the hypothalamus.

Function of copeptin

While the physiological function of AVP is well defined by its effects on tissue-specific receptors (V_{1a} receptor: mediates vasoconstriction and platelet aggregation; V_{1b} receptor: ACTH secretion; and V_2 receptor: water balance), the exact role of copeptin in normal physiology is unclear as it has no known receptors. In this regard, copeptin has been hypothesised to function as a chaperone for pre-proAVP release from the magnoceullar

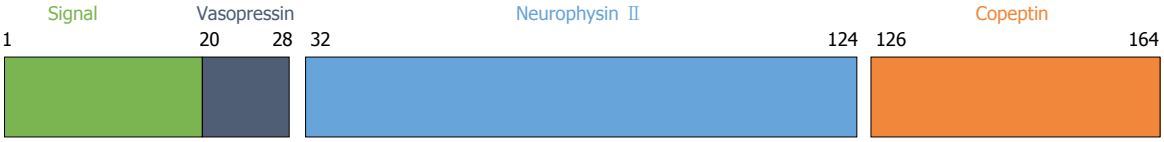

Figure 1 The precursor peptide of arginine vasopressin, also known as pro- arginine vasopressin. The indicative numbers of the amino acid positions of the peptide. Copeptin is the C-terminal peptide of pro-AVP and is released with AVP during precursor processing. Figure adapted from Reference[22]. AVP: Arginine vasopressin.

Table 1 Distinct production sites of precursor arginine vasopressin in hypothalamus

Site of synthesis in hypothalamus	Magnocellular neurons (Supra-optic and paraventricular)	Parvocellular neurons
Processing of AVP	Occurs during axonal transport in the infundibulum with copeptin and neurophysin acting as chaperones for correct AVP folding	Occurs in the parvocellular neurons where it released with other releasing hormones, such corticotrophin releasing hormone
Storage	Posterior pituitary	Hypothalamus
Stimuli for release	Osmotic and haemodynamic stimuli from the posterior pituitary gland	Released in response to humoural stress together with CRH, which both act on the adrenal gland to release cortisol

AVP: Arginine vasopressin.

Table 2 Summary of vasopressin limitations compared to copeptin advantages as a biomarker using the thermo scientific B.R.A.H.M.S KRYPTOR assay (adapted from Thermo Fisher scientific)

Features	Limitations of measuring AVP	Advantages of CT-proAVP (Copeptin)
Ex vivo stability	Unstable (even at -20 ℃)	Stable at > 3 d at room temperature
Sample volume required	400 L	50 μL
Time to results	3 working days	approximately 1 h
Sensitivity	Low (small molecule size, measured only by competitive immunoassay)	High (larger size, can be measured using a sensitive sandwich immunoassay)
Measuring range	1.15-73.8 pmol/L	0.7-500 pmol/L and up to 2000 pmol/L with automated dilution
Handling	Manual	Automatic

AVP: Arginine vasopressin.

neuron, but its role in peripheral tissues (if any) remains unknown. Thus, the literature has considered that copeptin is simply an inert biomarker with no direct role in the pathogenesis kidney cyst formation, but clearly further studies (such as gene knockout experiments in mice) are needed to evaluate this premise.

Degradation of copeptin

Another gap in knowledge is that little is known about the pharmacokinetics and degradation of copeptin. Available data suggests that it is rapidly cleared from the circulation either through the degradation by tissue-bound proteases, renal excretion and/or hepatic metabolism. New data on this topic are likely to emerge in the future and in this regard, a recent study reported that while the release of copeptin in response to elevation in the serum osmolality in healthy individuals was similar to AVP, its decay in the serum was two-fold longer than AVP[21]. Understanding the clearance of copeptin also has major implications for interpreting values in the setting of renal impairment, as reduced glomerular filtration is associated with elevated serum copeptin and thereby, confounds its value as a unique prognostic biomarker in ADPKD (as discussed below).

ADVANTAGES AND DISADVANTAGES OF COPEPTIN MEASUREMENT IN THE LABORATORY

It is well known that the laboratory testing of AVP is time-consuming and not practical (primarily because it is rapidly removed from the circulation with a half-life of less than 30 min requiring a large volume of serum) and is unstable in serum[22,23]. On the other hand, copeptin is released in equimolar amounts with AVP[24], is stable for up to 14 d in serum at room temperature; can be rapidly measured (0.5-2.5 h); and requires only 50 L of serum, making it potentially suitable for a high-throughput clinical pathology laboratory[12]. These analytical comparisons in methodology are summarised in Table 2.

The disadvantage of the copeptin assay is that in many countries, including in Australia, the most widely used and validated assay is a sandwich immunoluminometric method by ThermoFisher Scientific which requires purchase of a specific instrument[22]. As there is insufficient evidence for the use of copeptin in routine clinical practice, it is not currently provided by most clinical pathology laboratories and therefore, not readily

accessible. Other manufacturers other than Thermo-Fisher Scientific produce in-house ELISA kits[25] to measure copeptin, and this might make it easier to access the assay, but this is suitable for the clinical setting and large numbers of samples need assayed using a standardised technique. In sum, insufficient clinical evidence has not allowed further development of copeptin for clinical use in ADPKD, and the assay remains primarily restricted to the research setting.

MULTIPLE LIFESTYLE FACTORS INFLUENCE THE BASAL LEVELS OF SERUM COPEPTIN

Several lifestyle factors modify the basal level of copeptin in an individual. The most well studied variable is fluid intake. In healthy individuals, a chronic increase in total fluid intake by approximately 1 L/d above baseline reduced serum copeptin from 5.18 to 3.90 pmol/L[26]. Similarly, in patients with Stage 3 CKD (n = 28), the median copeptin level was 17 pmol/L and declined to 4.2 pmol/L with fluid intake over a 6-week period[27], confirming that: (1) Patients with CKD have higher levels of copeptin; and (2) increasing fluid intake can attenuate long-term copeptin levels. The type of fluid consumed may also influence the change in copeptin, as preclinical data in rats shows that rehydration with 20% hypertonic fructose increased plasma osmolality, AVP release and oxidative renal injury in rats with mild dehydration, whereas this effect was not seen with plain water[28].

Multiple other factors may influence the basal levels of copeptin. A cross-sectional analysis of the Groningen population-based cohort study (n = 6801) showed that in addition to low fluid intake, other dietary factors (high sodium, high protein and low potassium), alcohol intake and smoking were all associated with higher serum copeptin levels[29]. However, it is noteworthy that, in contrast to the Groningen study, alcohol suppresses the release of vasopressin from the pituitary gland[30,31]. Similarly, smoking is well known to stimulate the release of AVP in the blood plasma. In rabbits, an injection of 0.5 mg/kg of nicotine increased AVP concentrations by nearly 40 times[32]. The relative importance of each of these factors in influencing copeptin levels, and how they should be considered prior to collecting blood for copeptin in individuals, has not been standardized and further studies are required.

BASELINE SERUM COPEPTIN IS NOT DIAGNOSTIC OF ADPKD AND IS STRONGLY CORRELATED WITH RENAL FUNCTION

Several investigators have raised the hypothesis that serum AVP, and therefore copeptin, could be mildly elevated in ADPKD patients compared to the general healthy population[33]. For example, in a small study of 30 patients, the difference in mean serum copeptin was 8.92 pmol/L [inter-quartile range (IQR): 0.66-21.86] in ADPKD patients (eGFR 100 ± 23 mL/min/1.73 m^2) compared to 6.08 pmol (IQR 0.92-10.79) in healthy individuals (eGFR 104 ± 12 mL/min/1.73 m^2) (P = 0.22). This mild elevation is most likely due to subclinical volume depletion due to increased urinary losses as a result of impaired urinary concentrating ability and nephrogenic diabetes insipidus, as following water deprivation for 14 h, the difference in mean serum copeptin between ADPKD compared to healthy patients become statistically significant (ADPKD: 17.01 pmol/L; IQR 7.94-17.78 *vs* healthy: 7.75 pmol, IQR 3.81-8.80; P = 0.04)[33]. However, based on published studies, there is little evidence to support that these findings are specific or diagnostic of ADPKD. In particular, a recent study showed that: (1) Mean levels of copeptin in ADPKD patients were comparable to patients with other types of CKD, such as IgA nephropathy (ADPKD: 26.6 pmol *vs* IgA nephropathy: 20.7 pmol/L; P = 0.84)[34,35], and (2) the levels were more strongly correlated with the serum creatinine rather than the specific cause of CKD[35].

IS SERUM COPEPTIN A PROGNOSTIC BIOMARKER OF RENAL OUTCOMES IN ADPKD?

With this background, the remainder of this article will evaluate the specific utility of copeptin as a biomarker in ADPKD. The National Institutes of Health Biomarker Consortium defines a "biomarker" as a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions[36]. More specifically, the two sub-groups that are relevant to the discussion of copeptin and ADPKD are a "prognostic biomarker" (defined as biomarker that identifies the likelihood of a clinical event, recurrence or progression), and a "predictive biomarker" (defined as a biomarker that identifies those who are likely to respond to a treatment than those that are negative for a biomarker). Furthermore, Park and Ahn[37] outlined that the ideal biomarker in ADPKD should fulfil three characteristics: (1) It should correlate with the clinical severity of ADPKD; (2) it should detect patients at high-risk of progression; and (3) short-term changes should predict a clinical endpoint. In addition, validation is the process of assessing the biomarker and its measurement performance characteristics, and determining the range of conditions under which it provides reproducible and accurate data. The assessment of whether serum copeptin fulfils the criteria and validation in ADPKD is limited by the paucity of data, as only 26 articles were identified by a PubMed search using the terms "copeptin" and "ADPKD" (with at least

6 being review articles). Furthermore, many of these studies consist of small sample sizes. Despite this, the available data was analysed to answer two questions.

Are serum and urinary copeptin levels correlated with markers of disease severity?

Several cross-sectional studies show that serum copeptin is positively correlated with TKV and negatively correlated with eGFR. Furthermore, longitudinal studies show that higher copeptin levels had significantly higher TKV and urine osmolality, evidently shown by a study where TKV increased by 71% as copeptin levels increased by 23%[38,39]. Furthermore, a recent study also showed that serum copeptin predicted changes in fibromuscular dilatation in patients with ADPKD, an indicator of cardiovascular disease. To date, only one cohort has evaluated the role of urinary copeptin to creatinine ratio, which reported moderate correlations with ht-TKV (r = 0.383, P = 0.008) and eGFR (r = -0.304, P = 0.036) in 50 Japanese patients with ADPKD[40].

Are there any confounding factors that influence the interpretation of serum and urinary copeptin in ADPKD?

Several studies show that copeptin has a strong relationship with eGFR. Corradi *et al*[35] recently demonstrated that glomerular filtration affects copeptin to a greater extent rather than its correlation to AVP. The authors of a previous study indicated that after the removal of a kidney, the copeptin levels relatively remained stable, however, only GFR had declined by 40%[33,34]. A greater number of nephrons in the body would indicate a relatively higher AVP activity, and hence, a proportional increase in copeptin. The latter, however, opposes this theory, suggesting that perhaps copeptin is only a filtration marker rather than a disease severity marker for ADPKD. Furthermore, it is not known whether eGFR also confounds the level of urinary copeptin[40].

IS SERUM COPEPTIN A PREDICTIVE BIOMARKER OF TREATMENT RESPONSE TO VASOPRESSIN RECEPTOR ANTAGONISTS OR PRESCRIBED FLUID INTAKE IN ADPKD?

Several authors have suggested that higher baseline levels of copeptin could be used as a method to select patients for AVP blocking therapies, such as tolvaptan or prescribed fluid intake[12]. While this is an attractive hypothesis, it has yet to be formally tested and validated. In this regard, it would certainly be possible to perform a post-hoc analysis of the large randomized controlled trials involving tolvaptan to answer this question. Similarly, an ongoing randomized controlled trial of prescribed fluid intake will evaluate the long-term changes in serum copeptin and its effect on the progression of TKV[41].

CONCLUSION

There is strong interest in the role of copeptin as a molecular biomarker in ADPKD. While aligned with the pathogenesis of ADPKD, copeptin seems attractive for this purpose, but many questions remain. The most important question is whether measuring serum copeptin adds extra value over the standard clinical management tools (such as the PRO-PKD score, eGFR, TKV) to predict renal prognosis[42]? If so, does this predict whether patients with elevated copeptin are more likely to respond to therapies that suppress AVP (either or both pharmacological or life-style factors, such as fluid intake)? If these fundamental questions can be answered, other issues can be addressed, such as: What level of copeptin will be effective in attenuating renal cyst growth? How often should copeptin be measured? Should it be evaluated in all patients who present for the first time? What are the confounding factors that might influence interpretation of the data? Does copeptin itself have a direct pathological role in ADPKD? What is the value of measuring urinary copeptin, and how does serum copeptin compare with other markers of the AVP axis, such as urinary cAMP excretion?

Clearly, well designed prospective studies and health economic data will assist in answering these questions and evaluating the role of copeptin in the management of patients with ADPKD. Until this evidence is available, it will be difficult to influence policy-makers and regulatory bodies to utilize this test in the routine clinical care of patients with ADPKD.

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P- Reviewer: Aguiari G, Cheungpasitporn Wi, Hori T, Simone G
S- Editor: Cui LJ **L- Editor**: A **E- Editor**: Li RF

World Journal of Nephrology

Submit a Manuscript: http://www.f6publishing.com

World J Nephrol 2018 March 6; 7(2): 58-64

DOI: 10.5527/wjn.v7.i2.58

ISSN 2220-6124 (online)

MINIREVIEWS

Diabetic muscle infarction in end-stage renal disease: A scoping review on epidemiology, diagnosis and treatment

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Author contributions: Yong TY and Khow KSF designed the study, performed the research and wrote the paper.

Conflict-of-interest statement: The authors have no financial relationships to disclose.

Manuscript source: Invited manuscript

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Received: November 9, 2017
Peer-review started: November 10, 2017
First decision: December 13, 2017
Revised: December 25, 2017
Accepted: January 23, 2018
Article in press: January 23, 2018
Published online: March 6, 2018

Abstract

Diabetic muscle infarction (DMI) refers to spontaneous ischemic necrosis of skeletal muscle among people with diabetes mellitus, unrelated to arterial occlusion. People with DMI may have coexisting end-stage renal disease (ESRD) but little is known about its epidemiology and clinical outcomes in this setting. This scoping review seeks to investigate the characteristics, clinical features, diagnostic evaluation, management and outcomes of DMI among people with ESRD. Electronic database (PubMed/MEDLINE, CINAHL, SCOPUS and EMBASE) searches were conducted for ("diabetic muscle infarction" or "diabetic myonecrosis") and ("chronic kidney disease" or "renal impairment" or "dialysis" or "renal replacement therapy" or "kidney transplant") from January 1980 to June 2017. Relevant cases from reviewed bibliographies in reports retrieved were also included. Data were extracted in a standardized form. A total of 24 publications with 41 patients who have ESRD were included. The mean age at the time of presentation with DMI was 44.2 years. Type 2 diabetes was present in 53.7% of patients while type 1 in 41.5%. In this cohort, 60.1% were receiving hemodialysis, 21% on peritoneal dialysis and 12.2% had kidney transplantation. The proximal lower limb musculature was the most commonly affected site. Muscle pain and swelling were the most frequent manifestation on presentation. Magnetic resonance imaging (MRI) provided the most specific findings for DMI. Laboratory investigation findings are usually non-specific. Non-surgical therapy is usually used in the management of DMI. Short-term prognosis of DMI is good but recurrence occurred in 43.9%. DMI is an uncommon complication in patients with diabetes mellitus, including those affected by ESRD. In comparison with unselected patients with DMI, the characteristics and outcomes of those with ESRD are generally similar. DMI may also occur in

kidney transplant recipients, including pancreas-kidney transplantation. MRI is the most useful diagnostic investigation. Non-surgical treatment involving analgesia, optimization of glycemic control and initial bed rest can help to improve recovery rate. However, recurrence of DMI is relatively frequent.

Key words: Diabetic muscle infarction; Dialysis; End-stage renal disease; Kidney transplant; Renal replacement therapy

Core tip: Diabetic muscle infarction (DMI) is an uncommon complication in patients with end-stage renal disease, including kidney transplant recipients. Early recognition of DMI is vital to initiation of prompt treatment. Magnetic resonance imaging is the investigation of choice for diagnosing DMI. Non-surgical treatment involving analgesia, optimization of glycemic control and initial bed rest appears to improve recovery rate. However, recurrence of DMI is relatively common.

Yong TY, Khow KSF. Diabetic muscle infarction in end-stage renal disease: A scoping review on epidemiology, diagnosis and treatment. *World J Nephrol* 2018; 7(2): 58-64 Available from: URL: http://www.wjgnet.com/2220-6124/full/v7/i2/58.htm DOI: http://dx.doi.org/10.5527/wjn.v7.i2.58

INTRODUCTION

Diabetic muscle infarction (DMI) refers to spontaneous ischemic necrosis of skeletal muscle among people with diabetes mellitus, unrelated to atheroembolism or occlusion of major arteries. DMI is also referred to as spontaneous diabetic myonecrosis. Angervall and Sterner initially described DMI in 1965 as "tumoriform focal muscular degeneration"[1]. Although diabetes mellitus is a relatively common condition, DMI is a comparatively infrequent complication.

Diabetic nephropathy or other forms of chronic kidney disease are commonly seen in patients with DMI, affecting as many as 71%[2]. Of the diabetes-related microvascular complications, diabetic nephropathy is the most common one to be present among patients with DMI. Therefore, a proportion of DMI occurs in patients with end-stage renal disease (ESRD). However, little is known about the manifestation, diagnosis, management and outcomes of DMI in patients with ESRD.

This scoping review seeks to answer several questions about DMI regarding patient characteristics, clinical features, diagnostic evaluation, management and outcomes when this condition occurs in people with ESRD.

SEARCH STRATEGY AND STUDY IDENTIFICATION

The literature search was performed in July 2017 in four electronic databases. We searched MEDLINE/PubMed, CINAHL, SCOPUS and EMBASE with key words: ("Diabetic muscle infarction" OR "Diabetic myonecrosis"), AND ("Chronic kidney disease" OR "Renal impairment" OR "Dialysis" OR "Renal replacement therapy" OR "Renal transplant"). The search was done on publications between January 1980 and June 2017. Searches were limited to English language publications. Relevant references from articles identified from the search were also reviewed for comprehensive identification.

SELECTION OF STUDIES

This scoping review included cohort studies, case series and case reports with DMI in the setting of ESRD. Inclusion criteria include patients with ESRD requiring renal replacement therapy (RRT), clinical presentation of cases were adequately described, sufficient data on investigations, treatment and clinical outcome. The authors excluded studies that had not sufficiently eliminated trauma, inflammatory myositis, infection, neoplasm and deep vein thrombosis as possible aetiologies.

The selection process consisted of three stages. In the first stage, two reviewers (KK and TY) independently screened articles based on the title. In the case of doubt or disagreement, the articles were included in the abstract review stage. In the second stage, the abstracts of all articles are selected from the initial stage were reviewed and assessed by two reviewers (KK and TY). Any disagreement was resolved by discussion between the two reviewers. In the final stage, the remaining articles were fully reviewed using pre-determined inclusion criteria and assessed by two independent reviewers (KK and TY). Any disagreement between reviewers was solved by the two reviewers.

DATA EXTRACTION

A standardized template was used to extract data from the included studies using the following heading: general information such as title of article, main author and publication year, patient or cohort characteristics, type of diabetes mellitus, type of RRT (hemodialysis, peritoneal dialysis and kidney transplant), microvascular complications (retinopathy and peripheral neuropathy), macrovascular complications (coronary artery disease, ischaemic strokes and peripheral arterial disease) and pattern of muscle involvement. Laboratory and radiological investigation findings during initial evaluation or during recurrence were also obtained. Clinical outcomes to be considered in this review include time

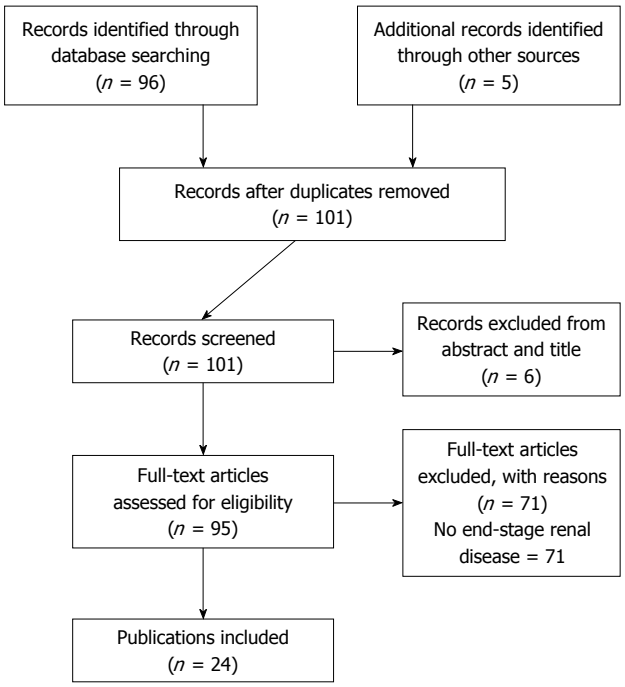

Figure 1 Flow diagram of the review and selection of publications.

to recovery from DMI, recurrence rate and death during the follow-up period.

We identified 101 articles from our initial search. Six were excluded because these were published in non-English languages (Figure 1). Twenty-four publications containing 41 cases with DMI in the setting of ESRD were eligible to be included in this review[3-26].

PATIENT CHARACTERISTICS

Of the 41 patients with DMI in the setting of ESRD, 22 occurred in women (53.7%). The mean age of presentation of all cases was 44.2 years (range: 19.0-67.0 years). Of these, 17 (41.5%) had type 1 diabetes, 22 (53.7%) had type 2 diabetes and 2 (4.9%) had cystic fibrosis related diabetes (Table 1). One report did not provide information about the type of diabetes that the patient had. The two patients with cystic fibrosis also had lung transplantation. The mean duration of diabetes mellitus at the time of DMI diagnosis was 17.3 (range: 10.0-30.0) years for type 1 diabetes and 15.7 (range: 3.0-24.0) years for type 2 diabetes. Glycohemoglobin (HbA_{1c}) values at the time of DMI diagnosis were reported in 18 cases and the average value was 7.2% (range: 5.0%-12.4%). Eleven out of 18 (61.1%) had HbA_{1c} above 7.0%.

All patients in this cohort had diabetic nephropathy. The RRT modalities included hemodialysis (25 patients; 59%), peritoneal dialysis (9; 22%), combined kidney-pancreas transplantation (4; 16%), kidney transplantation (1; 3%). For eight patients in whom information was provided, the mean duration of receiving dialysis was 2.1 years (range: 2 mo to 6 years). Twenty-four patients had concurrent retinopathy (58.5%) and 23 had peripheral neuropathy (56.1%). In terms of macro-vascular diseases, six were reported to have coronary artery disease and three had peripheral arterial disease.

CLINICAL FEATURES

The most frequently affected muscle groups were in the lower limbs (36; 87.8%), in comparison with the upper limbs which were reported only in five cases[11,22]. In the lower limb, DMI more commonly affected the proximal leg (24/36; 66.7% of those with lower limb involvement) than distal leg, *i.e.*, below knee (6/36; 16.7%). Six patients (16.7%) had both proximal and distal leg muscles involved. Most had unilateral (80.5%) involvement on initial presentation, while eight cases had bilateral involvement. Sixteen (39.0%) involved multiple muscle groups on initial presentation.

The most common clinical presentation of DMI was abrupt onset of pain in the affected muscle group (100%), accompanied by local swelling (97.6%). Fever was reported in six patients (14.6%). In the current analysis, compartment syndrome has been reported in one case[23].

INVESTIGATIONS

Results of laboratory investigations are usually non-specific for DMI. White cell count (WCC) values were reported in 35 cases but only 31.4% were elevated (Table 2). One patient was found to be leucopenic[15]. Inflammatory markers are commonly increased in DMI. C-reactive protein (CRP) was reported in 11 cases and was elevated in nine (81.8%) (Table 2). The mean CRP in these cases was 153.6 mg/L (range: 5.0-361.0 mg/L). Erythrocyte sedimentation rate (ESR) in nine cases and was increased in eight (88.9%) (Table 2). The mean ESR was 82.9 mm/h (range: 3.0-137.0 mm/h).

Creatine kinase (CK) values were reported in 34 cases but was elevated in 50.0% (considered as greater than 150.0 IU/L for females or 250.0 IU/L for males) (Table 2). When measured on initial presentation, the mean absolute CK was 292.9 IU/L (range: 23.0-1066.0 IU/L).

Magnetic resonance imaging (MRI) was performed in most patients (35/41; 85.4%). The common abnormalities observed on MRI is muscle enlargement (33/35; 94.2%) and muscle oedema with hyperintense T2 signal (30/35; 85.7%) (Table 2). Other findings include subcutaneous edema (17/35; 48.6%).

Ultrasound was performed in 28 (68.3%) patients but all were done to exclude deep vein thrombosis. Subcutaneous or muscle edema on ultrasound was reported in six cases (21.4%). Computed tomography (CT) was performed less frequently among cases included in this review (12/41; 29.3%).

Muscle biopsy was performed in 16 cases (39.0%). Biopsies usually demonstrate muscle necrosis (100%) and inflammatory cell infiltration (87.5%) (Table 2). In some cases, muscle fibre regeneration (43.8%) was

Table 1 Characteristics of patients with diabetic muscle infarction in the setting of end-stage kidney disease and a cohort from Horton *et al*'s systematic review

	Current review (n = 41)	Findings from Horton *et al*[2] (n = 126)
Mean age (range), yr	44.2 (19.0-67.0)	44.6 (20.0-67.0)
Female/male, *n* (%)	22 (53.7)/19 (46.3)	68 (54.0)/58 (46.0)
Type of diabetes		
Type 1, *n (%)*	17 (41.5)	54/108 (50.0)
Type 2, *n (%)*	22 (53.7)	45/108 (41.7)
Cystic-fibrosis-related, *n (%)*	2 (4.9)	2/108 (1.9)
Concurrent diabetes-related complications		
Retinopathy, *n (%)*	24 (58.5)	83 (65.8)
Neuropathy, *n (%)*	22 (56.1)	-
Coronary artery disease, *n* (%)	6 (14.6)	-
Peripheral arterial disease, *n* (%)	3 (7.3)	-
Type of renal replacement therapy		
Hemodialysis, *n* (%)	25 (60.1)	-
Peritoneal dialysis, *n* (%)	9 (21.9)	-
Pancreas-kidney transplantation, *n* (%)	4 (9.8)	-
Kidney transplantation only, *n* (%)	1 (2.4)	-
Pattern of muscle involvement		
Lower limbs	36 (66.7)	-
Proximal lower limbs (above knee)	24 (58.5)	90 (71.2)
Distal lower limbs (below knee)	6 (14.6)	19 (15.3)
Both proximal and distal lower limbs	6 (14.6)	-
Upper limbs	5 (12.2)	7 (5.4)
Unilateral limb	33 (80.5)	-

Table 2 Investigation results in patients with diabetic muscle infarction in the setting of end-stage renal disease and a cohort from Horton *et al*'s systematic review

	Present review		Findings from Horton *et al*[2]
	Number of patients who had the investigation	*n* (%)	*n/n* (%)
Leucocytosis (WCC > 11.0 × 10^9 cells/L)	35	11 (31.4)	48/112 (42.5)
Leucopenia (WCC < 4.0 × 10^9 cells/L)	35	1 (2.9)	-
Elevated CRP (> 10 mg/L)	11	9 (81.8)	27/30 (90.0)
Elevated ESR (> 20 mm/h)	8	9 (88.9)	50/60 (83.3)
Elevated creatine kinase (> 150 IU/L for females; > 250 IU/L for males)	34	17 (50.0)	31/98 (31.6)
HbA_{1c} > 7.0%, *n* (%)	18	11 (61.1)	-
MRI findings		35 (85.4)	
Muscle enlargement	35	33 (94.2)	-
Muscle edema	35	30 (85.7)	76.8
Subcutaneous edema	35	17 (43.6)	-
Muscle biopsy findings		16 (39.0)	
Muscle necrosis	16	16 (100)	-
Inflammatory cell infiltration	16	14 (87.5)	-
Muscle fibre regeneration	16	7 (43.8)	-

CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; MRI: Magnetic resonance imaging; WCC: White cell count.

also present.

MANAGEMENT

Treatment details were provided in 36 cases. Most patients (31/36; 86%) were managed with analgesia. Four patients received aspirin therapy. There was no clear description in most cases as to whether patients rested in bed or received physiotherapy. Five patients (13.8%) required surgical intervention, which involved debridement of the affected muscle.

In a small number of patients, intensification of glycaemic control (four patients) and dialysis (one patient) were reported. Seven patients received other forms of treatment which included antibiotics, prednisolone and erythropoietin therapy. However, the effectiveness of these treatments could not be ascertained in this review due to the limited numbers.

Outcomes

As the management approaches to DMI vary considerably in between patients, the efficacy of each treatment modality or the comparisons of outcomes could not be evaluated in this small sample size. In 15 patients, the time interval between diagnosis and resolution of the initial presentation of DMI was 1.4 mo

(range: 2 wk to 4 mo).

Recurrent muscle infarctions were reported in 43.9% of cases. None of the pancreas-kidney transplant recipients experienced any recurrence of DMI. Of those with recurrence of DMI, the mean time interval between the initial episode and the first recurrence was 5.3 mo (range: one week to 18 mo). Most of the recurrences in this case series occurred in a different muscle group to the initial episode (9/11; 81.8%).

In the cases reviewed, seven patients died during the follow-up period after developing DMI. Five of these patients were receiving haemodialysis and two were on peritoneal dialysis. No death was reported among kidney transplant recipients with DMI.

DISCUSSION

DMI is a rare disorder encountered in patients with longstanding diabetes mellitus and end-organ complications. Among patients with ESRD, the manifestation of DMI, investigation findings, management approach and rate of recurrence are similar to the other patients without ESRD who develop DMI. However, this study was not designed to estimate the incidence of DMI among patients with ESRD.

In a review by Trujillo-Santos *et al*[27] of 166 episodes of DMI in 115 patients, the mean age at the time of presentation was 42.6 years. The mean duration between diagnosis of diabetes mellitus and first episode of DMI was 14.4 years. In the present review, those with DMI in the setting of ESRD also have a similar age at the time of presentation and duration of diagnosis with diabetes mellitus. As this was a review of case report and series, it is not possible to determine if there is any difference in incidence of DMI between type 1 and type 2 diabetes. Similarly, any difference in incidence between patients who receive hemodialysis and peritoneal dialysis cannot be established in this review. Nonetheless, this review indicates that DMI can occur in patients after kidney transplantations including combined pancreas and kidney transplantations. Unsurprisingly, most patients have other concurrent diabetes-related microvascular complications such as retinopathy and peripheral neuropathy. In relation to patient characteristics, the current cohort of patients with ESRD and DMI was relatively similar to an unselected group of patients with DMI[2].

There is a propensity for DMI to occur among patients with longstanding diabetes mellitus whose glycaemic control has deteriorated over time. More than 60% of ESRD patients who develop DMI have HbA_{1c} of greater than 7.0%, which indicated inadequate glycaemic control. DMI has been reported in a number of cases with poor glycaemic control[28]. The pathophysiology for DMI is still unknown but several hypotheses have been suggested. Atherosclerotic changes, diabetes-related microangiopathy, vasculitis with associated thrombosis, and ischemia-reperfusion injury have been postulated as possible mechanism for DMI[29]. The thromboembolic mechanism is thought to involve endothelial damage resulting in tissue ischemia which leads to muscle injury and ischaemic necrosis. Reperfusion of ischaemic muscles lead to increase in oxygen radicals and reduced nitric oxide, which in turn releases inflammatory mediators. Alterations in the coagulation-fibrinolysis system resulting in damage of vascular endothelium and hypercoagulability have also been suggested as part of the pathophysiology of DMI[30].

The diagnosis of DMI remains challenging and can lead to underdiagnosis[31]. At present, the diagnosis of DMI involves the combination of clinical assessment, MRI and muscle biopsy with atypical presentations. DMI is characterized by localized muscular pain, swelling and tenderness and there is a more frequent involvement of the lower limb musculature, especially the thigh. Rare cases of upper limb muscle involvement have been reported[11,22]. The reason for this predilection for lower limb muscles is uncertain.

Although MRI findings are not pathognomonic for DMI, this modality is still the most sensitive and specific for diagnosis of this condition[2]. Characteristic features on MRI include muscle enlargement, oedema within affected muscles, subcutaneous and interfascial oedema[32]. On MRI examination, the differential diagnoses include intramuscular abscess, inflammatory myositis and necrotising fasciitis[27]. Sometimes it can be difficult to distinguish these entities and muscle biopsy may be required. Other imaging modalities such as ultrasound or CT scans may assist in excluding other disorders but may not enable the correct diagnosis of DMI. In patients with ESRD, calciphylaxis or calcific uremic arteriolopathy (CUA) also needs to be considered in the differential diagnosis. CUA is characterized by vascular and other soft tissue calcification, intimal hypertrophy, and thrombosis of small vessels that results in painful tissue necrosis, including skeletal muscle[33]. The ischaemic injury in DMI is more confined to the muscle whereas in CUA, cutaneous and other soft tissues can be affected. A histopathological feature of CUA will often reveal extensive calcification, microthrombosis and endovascular fibrosis of small subcutaneous arteries leading to cutaneous ischemia whereas the calcification is not a prominent feature of DMI[33].

Muscle biopsy can lead to a conclusive diagnosis but this is not currently recommended because of the risk from procedure-related complications[34]. It can be used as a diagnostic tool in atypical presentations of DMI. Histopathological changes in DMI can vary depending on the timing of biopsy. In general, DMI presents as a pale nonhemorrhagic muscle. Initially, light microscopy would usually show muscle necrosis and phagocytosis of necrotic muscle fibres. At a later stage, the necrotic muscles are replaced by fibrous tissue, lymphocyte infiltration and muscle regeneration.

Blood investigations are generally non-specific for DMI. Surprisingly, a rise in CK has only been reported in half of the patients with DMI and ESRD. It is possible that the lack of rise in CK may be related to the delay in some patients seeking attention and measurement is

performed after this marker has peaked. This is relatively consistent with other systematic review of unselected patients with DMI. CRP and ESR were commonly elevated in DMI but these markers were not specific for this condition.

Early recognition of DMI is important for prompt initiation of treatment. Among patients with DMI and ESRD, supportive care consisting of analgesia was the main management approach. Generally, patients with DMI also receive intensive glycaemic control. Some clinicians have recommended avoiding physical therapy during the acute phase of DMI because it can prolong recovery but others have not observed this[4,35]. Surgical intervention is required only in selected cases but this is not first-line option because of association with a more prolonged recovery[14]. Other treatment modalities such as glucocorticoid, antibiotics and erythropoietin have been tried but their efficacy in DMI is uncertain.

The short-term prognosis of DMI is good and most patients recover within 6 mo but recurrence is frequent among patients with ESRD. Almost 50% will experience recurrence of DMI, mostly in another muscle group, within 6 mo after the initial episode. In comparison to unselected patients with DMI, the recurrence rate was reported at 29.2%[2]. It was interesting to observe that no recurrence has been reported among transplant recipients. Further research is needed to confirm this observation. The long-term survival of patients with DMI can be variable. Death within a year by an episode of DMI are often related to other end-organ complications present in these patients.

The present review is limited by the use of case reports or series with the largest one having four patients. Not all patients had the same type of investigations for DMI. There are also limited reports about functional impairment experienced by patients during episodes of DMI. Furthermore, follow-up of patients in the included reports varied considerably.

More research is needed to better understand the pathogenesis of DMI, strategies for effective treatment and prevention of recurrence. More specifically, in the setting of ESRD and RRT, more investigation is needed to ascertain if adequacy of the RRT may influence outcomes. It will also be useful to better understand the natural history of DMI among patients who have had renal transplantation or those who are candidates for transplantation.

CONCLUSION

DMI is an uncommon complication of diabetes mellitus that can also affect patients with ESRD who are receiving dialysis or recipients of kidney transplantation. Clinical findings which supported by MRI examination is currently the best approach to confirm the diagnosis of DMI. Muscle biopsy is not recommended but may be required in atypical presentations. The best management approach for DMI is currently unclear and present practice is still largely based on expert opinion because of limited evidence. Nonetheless, nonsurgical treatment appears to be a better option than surgical intervention. The short-term prognosis of DMI is good but recurrence is frequent.

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P- Reviewer: Kute VBB, Lai S, Stavroulopoulos A, Sureshkumar KK
S- Editor: Cui LJ **L- Editor**: A **E- Editor**: Li RF

World Journal of Nephrology

Submit a Manuscript: http://www.f6publishing.com

World J Nephrol 2018 March 6; 7(2): 65-70

DOI: 10.5527/wjn.v7.i2.65

ISSN 2220-6124 (online)

ORIGINAL ARTICLE

Basic Study

Genetic defects in ciliary genes in autosomal dominant polycystic kidney disease

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Author contributions: Skalická K and Kovács L substantially contributed to the design of the study; Skalická K, Hrčková G, Vaská A and Baranyaiová A performed the research and analyzed of data; all authors drafted the article and approved the final version of the article to be published.

Supported by Slovak Research and Development Agency under Contract, No. APVV-14-0234.

Institutional review board statement: Our study was approved by the Children's University Hospital Ethics Committee and informed consent was provided by all patients at the inception of the study.

Conflict-of-interest statement: The authors have declared that no conflict of interest exists.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: In our study, ARRIVE Guidelines have been adopted.

Manuscript source: Unsolicited manuscript

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Received: December 12, 2017
Peer-review started: December 13, 2017
First decision: December 27, 2017
Revised: December 31, 2017
Accepted: February 4, 2018
Article in press: February 4, 2018
Published online: March 6, 2018

Abstract

AIM

To evaluate the genetic defects of ciliary genes causing the loss of primary cilium in autosomal dominant polycystic kidney disease (ADPKD).

METHODS

We analyzed 191 structural and functional genes of the primary cilium using next-generation sequencing analysis. We analyzed the kidney samples, which were obtained from 7 patients with ADPKD who underwent nephrectomy. Each sample contained polycystic kidney tissue and matched normal kidney tissue.

RESULTS

In our study, we identified genetic defects in the 5 to 15 genes in each ADPKD sample. The most frequently identified defects were found in genes encoding centrosomal proteins (*PCM1*, *ODF2*, *HTT* and *CEP89*) and kinesin family member 19 (*KIF19*), which are important for ciliogenesis. In addition, pathogenic mutations in the *PCM1* and *KIF19* genes were found

in all ADPKD samples. Interestingly, mutations in the genes encoding the intraflagellar transport proteins, which are the basis of animal models of ADPKD, were only rarely detected.

CONCLUSION

The results of our study revealed the actual state of structural ciliary genes in human ADPKD tissues and provided valuable indications for further research.

Key words: Polycystic kidney disease; Primary cilium; Ciliary genes; Next-generation sequencing; Genetic variants

Core tip: Many studies have confirmed that the loss of primary cilia promotes renal cyst formation in autosomal dominant polycystic kidney disease (ADPKD). However, these studies are based on mouse models by the inactivation of various ciliary genes, and the actual status of these genes in human ADPKD tissues is unknown. In our study, we analyzed genetic defects in ciliary genes in the human polycystic kidney tissues and matched normal kidney tissues by next-generation sequencing. We found that the loss of the primary cilia in the human ADPKD tissues may be predominantly caused by defects of centrosomal proteins and KIF19 protein.

Skalická K, Hrčková G, Vaská A, Baranyaiová Á, Kovács L. Genetic defects in ciliary genes in autosomal dominant polycystic kidney disease. *World J Nephrol* 2018; 7(2): 65-70 Available from: URL: http://www.wjgnet.com/2220-6124/full/v7/i2/65.htm DOI: http://dx.doi.org/10.5527/wjn.v7.i2.65

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a multisystem disorder characterized by the formation of cysts in the kidneys and other organs. ADPKD affects more than 12.5 million people worldwide[1]. Approximately 50% of patients with ADPKD have end-stage renal disease (ESRD) by 60 years of age. Dialysis and kidney transplantation are the only treatment options for patients with ESRD[2]. The reason for the absence of targeted treatment is insufficient understanding of the molecular mechanism of cystogenesis.

The molecular nature of the disease includes germline mutation of either the *PKD1* (polycystin-1), *PKD2* (polycystin-2) or *GANAB* gene[3,4]. Although the germline mutation is present in every cell of the body, formation of cysts is limited to a small number of nephrons. This means that the germline mutation is not in itself sufficient to produce cysts; a second somatic event is also required[5]. More evidence has accumulated in recent years showing that the primary cilium plays an important role in the development of ADPKD.

The primary cilium is a signaling organelle that extends from the surface of the plasma membrane of most mammalian cells. Assembly of cilia depends on cell cycle progression, because the centrioles regulating the cell cycle are essential components in the formation of the basal body of the cilia[6]. The maintenance and elongation of cilia is ensured by a process called intraflagellar transport (IFT). In this process, protein complexes known as IFT trains carry cargo along tracks that run along the length of the cilia. Different motor proteins power the IFT trains in different directions. Kinesin moves IFT trains from the base to the tip, while dynein moves them back in the opposite direction[7].

Many studies have shown that the loss of primary cilia promotes renal cyst formation *in vivo*[8,9]. However, the epithelial cells of renal cysts are characterized not only by an absence of cilia but also by excessively long cilia[10-12]. Recent studies have confirmed that kidney cysts occurred following inactivation of polycystins in otherwise intact cilia or following complete removal of cilia by inactivation of IFT proteins. In addition, a relationship was shown between cilia and polycystins that regulates the severity of ADPKD. According this model, the progression of cysts is regulated by the duration of the interval time between the initial loss of polycystins and the subsequent ablation of cilia. These studies identified a new mechanism of cystogenesis based on the evidence that the loss of renal cilia inhibits cyst growth if the cilia are disrupted at the same time as the polycystins[13,14]. In addition, it was shown that defective primary cilia or the inactivation of ciliary genes induces aberrant signaling pathways associated with proliferation, differentiation and development in various PKD mouse models[13-16]. The structural deformity or absence of primary cilia is thus a key driving force in the development of ADPKD.

The results of these studies were achieved in mouse models by targeted inactivation of various ciliary genes, especially genes encoding IFT proteins. However, the actual mutation profile of ciliary genes in human ADPKD tissues is unknown. The aim of our study was to identify the mutation state of genes encoding the structural ciliary components in tissues from patients with ADPKD obtained by nephrectomy through targeted next-generation sequencing analysis.

MATERIALS AND METHODS

Sample collection

In our study, we obtained archived FFPE samples of polycystic kidney tissues and matched normal controls from 7 patients with ADPKD (four women and three men) who had undergone radical nephrectomy. Four patients had nephrectomy for the occurrence of complications associated with the enlarged kidney involved the recurrent infection of the urinary tract, arterial hypertension, and chronic pain. Three patients required nephrectomy to provide the space for the

kidney allograft. The FFPE samples were subjected to histopathological examination. The samples containing at least 50% epithelial cysts were considered suitable for genetic analysis. The genetic testing of the *PKD1* and *PKD2* genes had been carried out previously in our laboratory. The mutation in the *PKD1* gene was confirmed for all of the patients. Our study was approved by an ethics committee and informed consent was provided by all patients at the inception of the study.

DNA extraction and sample quality control

DNA was extracted from the FFPE tissues using the commercially available blackPREP FFPE DNA kit (Analytik Jena, Germany) and from peripheral blood using the QIAamp DNA Mini kit (Qiagen, Valencia, California, United States) according to the manufacturer's instructions. The extracted DNA specimens from FFPE were further quantified using the Qubit dsDNA HS assay kit (Life Technologies/Fisher Scientific, United States) and the Agilent NGS FFPE QC Kit according to the manufacturer's instructions.

Design of the gene panel

Candidate genes were selected using a gene list in the SysCilia Gold Standard (SCGC) Version 1 database[17]. A panel of 191 genes was designed using the web-based tool SureDesign (Agilent Technologies, United States). The regions of interest included coding regions with 10 base pair (bp) upstream and 10 bp downstream for capture of splicing donor and acceptor sites. Overall, we analyzed genes encoding the proteins of basal bodies (31 genes), centrioles (25 genes), centrosomes (22 genes), IFT (24 genes), transition zone (17 genes), axoneme (9 genes), ciliary membrane (8), cilioplasma (5 genes), ciliary proteins located in the nucleus (5 genes), plasma membrane proteins (5 genes), ciliary tip (4 genes), regulatory ciliary proteins in the Trans-Golgi network (3 genes), ciliary root (2 genes) and ciliary proteins with non-specific localization (31 genes).

Library preparation and variant calling

Sequencing libraries were prepared using the Agilent SureSelect^{XT} Custom 0.5 Mb up to 2.9 Mb according to the manufacturer's instructions. The sequencing analyses were performed on the HiSeq 2500 sequencing system (Illumina, United States). Data were analyzed using a software package that was commercially available - NextGENe™ (SoftGenetics, United States). Only variants with a minimum of 50-fold coverage for at least 80% of the targeted bases were included into analysis. Variants were marked as potential errors if they exhibited strong strand bias (< 0.10), low depth of coverage (< 10), low-quality score (< 30) or low average quality (< 2.0). The functional analysis of variants was performed by using Geneticist Assistant software (SoftGenetics, United States). This software used combined computational prediction methods (SIFT, Polyphen2, LRT, MutationTaster, ANNOVAR, FATHMM, CADD and Mutation Assessor) to calculate the mean pathogenicity score of identified variants. The pathogenic variants detected by NGS were verified by using the Sanger sequencing method. The germline origin of the pathogenic variants was excluded by analysis of DNA extracted from peripheral blood.

RESULTS

Analysis of sequencing data

In our study, we achieved a high quality of sequenced data. The mean total reads generated per sample was approximately 5000000, with more than 98% of reads aligned with the reference genome and more than 80% of reads mapping to targeted regions. In addition, 93% of the targeted regions were covered by more than 30 reads. Overall, 1440 variants were identified in 7 ADPKD samples. These variants represent disease-specific genetic changes without occurrence in matched normal kidney tissues. Based on the results of the functional analysis, 908 variants were classifying as benign. These variants were reported in known databases of somatic mutations and single nucleotide polymorphisms. Of these, 732 variants were classified as clearly benign. The remaining 176 variants were of unknown significance, but with high value of minor allele frequency, on the basis of which they were determined as polymorphisms.

The other 532 identified variants represented novel genetic changes without record in databases. The results of the functional prediction determined 52 variants as clearly pathogenic. For the other 480 variants, benign status was clearly confirmed in all predictive software. Identified pathogenic variants were present in 39 genes encoding the various structural components of the primary cilium. An overview of these genes is shown in Table 1. The most common pathogenic variants affected the proteins of basal bodies (10 genes), centrosomes (7 genes), centrioles (6 genes) and ciliary proteins with non-specific localization (5 genes). The proteins in other parts of the cilium were rarely mutated.

Analysis of disease-related pathogenic variants

The pathogenic variants were further analyzed based on their occurrence in individual ADPKD samples. The mutation profile of each analyzed sample was unique. Each sample had identified pathogenic variants in 5 to 15 genes, which varied from each other. However, the pathogenic variants in 5 genes were present in the vast majority of the ADPKD samples. Of these, 4 included genes encoded the centrosomal protein HTT (Huntingtin), the subdistal appendage of centriole ODF2 (outer dense fiber protein 2), the distal appendage of centriole CEP89 (centrosomal protein of 89 kDa) and a component of centriolar satellite PCM1 (pericentriolar material 1). The most affected gene was *PCM1*, in which pathogenic variants were present in all samples of ADPKD. The pathogenic variants in the *HTT*, *ODF2* and *CEP89* genes were identified in 5 of the 7 ADPKD samples. Another gene whose pathogenic variants affected all of the ADPKD samples was the *KIF19* (Kinesin

Table 1 Summary of genes affected by pathogenic variants and localization of their coding proteins

Localization	Genes affected by pathogenic variants
Basal body	*ODF2, AZI1, BBS12, BBS2, C21orf2, FLNA, LZTFL1, OFD1, PDYD7, TRAF3IP1*
Centrosome	*CEP89, HTT, CEP135, CEP290, MDM1, NINL, TRAPPC9*
Centriole	*PCM1, PIBF1, CBY1, FBF1, NEK1, SCLT1*
Unspecific localization	*STX3, PKD1L1, TTLL3, WDR60, CCDC35*
Ciliary tip	*KIF19, GLIS2*
Intraflagellar transport	*IFT172, IFT80*
Transition zone	*NUP37, NUP62*
Regulatory proteins	*RAB11FIP3, TRIP11*
Ciliary membrane	*CRB3*
Ciliary root	*CROCC*
Axoneme	*TMEM67*

Table 2 Description of the most common pathogenic variants identified in autosomal dominant polycystic kidney disease samples

Gene	DNA-level	Protein-level	Exon	Number of samples with mutation
PCM1	c.3423dupC	p.Ser1142Glnfs*7	22	7
KIF19	c.49dupC	p.Arg17Profs*20	2	7
CEP89	c.412_413delAA	p.Lys138glyfs*16	4	5
HTT	c.108_110delGCA	p.Gln2643del	1	5
ODF2	c.1118delT	p.Leu373Tyrfs*80	10	5

Family Member 19) gene. This gene encodes a key regulator of ciliary length located on the very top of the primary cilia in the ciliary tip included in anterograde IFT. In addition, we identified only one type of the pathogenic variant in these 5 genes (Table 2). In most cases, it was a frameshift mutation resulting in a premature stop codon and truncation of protein. According to the results of the predictive software, all detected variants negatively affected the function of the encoded proteins.

Interestingly, the pathogenic variants in genes encoding proteins of retrograde IFT and other structural ciliary genes were detected in only one of the ADPKD samples. The coverage of protein-coding regions and the flanking regions of all analyzed IFT genes showed high values in each of the ADPKD samples.

DISCUSSION

In the present study, we identified genetic changes in the structural ciliary genes in human ADPKD tissues. The most frequently affected genes encoded the centriolar and centrosomal proteins PCM1, ODF2, HTT and CEP89, which are essential for ciliogenesis. Recent studies have confirmed that the loss of these proteins specifically blocks ciliogenesis at the step of centriole-to-membrane docking. Undocked centrioles lose the signs of cilia assembly, even when they were previously under the influence of signals that support ciliogenesis[18,19]. Thus, disruption of the function of these genes may be the cause of cilia loss in the epithelial cells of renal cysts. The most commonly affected gene in this group was *PCM1*, which is also essential for the correct localization of several centrosomal proteins and for anchoring microtubules to the centrosome. It is generally known that centrosome dysfunction is linked to aneuploidy and chromosomal instability[20]. Many studies have shown increased incidence of chromosome imbalances and abnormal chromosome segregation in ADPKD tissues[21,22]. Loss of function of the *PCM1* gene may therefore be a key factor in these processes.

Another pathogenic variant, which was present in all of the analyzed ADPKD samples, was identified in the *KIF19* gene. This gene encodes a member of the kinesin superfamily protein included in anterograde IFT, which is localized to ciliary tips. The results of a recent study have provided evidence that KIF19 is a key determinant of the optimal length of cilia. The length of cilia was abnormally extended by knockdown *Kif19* in a mouse model[23]. Many studies have revealed that the change in the length of the primary cilium is an important trigger of various pathological processes in ADPKD[24,25]. The epithelial cells of renal cysts usually show an absence or shortening of the primary cilium. However, stages of interstitial fibrosis and end-stage renal disease are associated with the elongation of the primary cilia[26,27]. In our study, we analyzed the mutation profile in the nephrectomized ADPKD tissues withdrawn at the end stage of renal disease. Given that we identified the pathogenic variant of the *KIF19* gene in all analyzed ADPKD tissues, it may represent a significant event in the progression of the disease. However, these claims will require further analysis.

An interesting result of our study was the low frequency of mutations in the genes encoding IFT proteins. Kidney cysts arise in most mouse models following the disruption of cilia by targeted inactivation of genes encoding IFT components such as the heterotrimeric kinesin components KIF3a and the IFT proteins IFT20 and IFT88. However, genetic changes in these genes were not present in any analyzed ADPKD sample. We confirmed the presence of the pathogenic variants in only two genes (*IFT172* and *IFT80*) of the total number

of analyzed IFT genes. In addition, the pathogenic variants in these genes were detected in only one ADPKD sample. The results of our study showed that the loss of the primary cilia in the human ADPKD tissues may be predominantly caused by defects of centrosomal proteins and KIF19 protein. However, this claim requires confirmation by functional analysis of the use of animal models. It is also necessary to verify the results by analyzing a larger number of samples.

In our study, we identified the simultaneous occurrence of genetic changes in various ciliary genes. Each ADPKD tissue had pathogenic variants in 5 to 15 genes. The occurrence of multiple structural defects of the primary cilium may be due to several factors. Firstly, we analyzed the tissues at an advanced stage of the disease, in which genetic changes could have been accumulated. However, disturbances of centrosomal proteins may also be the cause of the multiple defects in the ciliary structure. An interesting finding was the presence of only one pathogenic variant in each individual gene. To determine whether they are "hotspot" mutations representing secondary somatic events in the development of the disease will require further analysis.

The results of our study may be limited by the relatively small number of analyzed samples. The reason for this small number is the fact that the majority of ADPKD patients do not require native nephrectomy, and cystic kidneys are not generally biopsied for technical and ethical reasons. However, this is the first study examining the complex mutational status of the structural and functional ciliary genes in human ADPKD tissues.

In conclusion, our study revealed genetic defects of ciliary genes that can lead to loss of the primary cilium in human ADPKD tissues. In addition, we identified unique genetic findings associated with the disease, which may play a significant role in the pathogenesis of the disease. However, other functional analyses are necessary to confirm this hypothesis. The results of our work thus provided valuable indications for the direction of further research in the area of molecular pathogenesis of ADPKD.

ARTICLE HIGHLIGHTS

Research background

The primary cilia and polycystins plays an important role in the regulating the severity of autosomal dominant polycystic kidney disease (ADPKD). While the loss of cilia or polycystins alone results in the development and progression of renal cysts, renal cilia involution reduces the progression of cyst growth induced by the inactivation of polycystins. The epithelial cells of renal cysts usually show various structural deformities of the primary cilium involve an absence, shortening or elongation. These structural changes can be caused by genetic defects of ciliary proteins. Mutation profile of ciliary genes in human ADPKD tissues is unknown. Revealing a genetic basis for ciliogenesis defects may identify causative factors of disease progression and the potential molecular targets for the development of new therapies of ADPKD.

Research motivation

Genetic defects of various ciliary genes whose inactivation leads to the development and progression of ADPKD have been identified in mouse models. However, recent studies have confirmed that the animal models of ADPKD incompletely mimic the human disease. Therefore, it is important to detect genetic abnormalities that can affect ciliogenesis directly in ADPKD human tissues.

Research objectives

The main objectives of this study is to identify the genetic defects of ciliary genes causing the loss of primary cilium in ADPKD human tissues. The results of our study are important indicators for directing further analysis.

Research methods

In our study, we analyzed 191 structural and functional ciliary genes using next-generation sequencing analysis. The tissue samples used in this study were obtained from 7 patients with ADPKD who underwent nephrectomy. Each sample contained polycystic kidney tissue and matched normal kidney tissue. All analyzed samples were formalin-fixed and paraffin-embedded. The germline origin of the identified variants was excluded by analysis of DNA extracted from peripheral blood.

Research results

We identified unique of mutation profile in each of analyzed ADPKD samples, which was characterized by the presence of pathogenic variants in 5 to 15 ciliary genes. The most frequently identified defects were found in genes encoding centrosomal proteins and kinesin family member 19, which are important for ciliogenesis. In addition, pathogenic variants in the *PCM1* and *KIF19* genes were found in all ADPKD samples.

Research conclusions

Our study had found that the human ADPKD tissues are characterized by the presence of several genetically altered ciliary proteins that plays an important role in ciliogenesis. The structural and functional disturbance of the primary cilium can be induced by mutations of these proteins. An interesting finding of our study was that the mutations in genes encoding the proteins of intraflagellar transport (IFT) were rarely mutated. These genes are considered candidate genes related to ADPKD in mouse models. Centrosomal proteins and kinesin family member 19 are the most commonly mutated ciliary proteins in renal epithelial cells derived from human ADPKD cysts. Consistent with finding of recent studies, we can confirm that animal models not completely mimic the human disease. Mouse models induce polycystic kidney disease by genetic inactivation of one ciliary gene especially genes encoding IFT proteins. However, the genes encoding the proteins of IFT are rarely mutated in the human renal cystic cells. This study offered new insight into comprehensive mutation profile of ciliary genes in human ADPKD tissues. The results of our study suggested that the loss of the primary cilia in the human ADPKD tissues may be predominantly caused by defects of centrosomal proteins and kinesin family member 19. The defects of centrosomal proteins may be also the cause of chromosome imbalances, which are often present in human ADPKD tissues. The genetic defects of the *KIF19* gene may be cause of the primary cilium elongation, which is a characteristic feature of the end-stage renal disease. This is the first study used of archived formalin-fixed and paraffin embedded tissues (FFPE) of ADPKD in order to determine mutation profile of ciliary genes by next-generation sequencing methods. An interesting finding was the presence of only one somatic pathogenic variant in each individual ciliary gene. To determine whether they are "hotspot" mutations representing secondary somatic events in the development of the disease will require further analysis. Somatic mutations in genes encoding centrosomal proteins and KIF19 were present in all analyzed ADPKD samples. These mutations were present exclusively in polycystic kidney tissues and did not occur in matched control so we can assume their effect on cystogenesis. If our hypotheses will be confirmed by further studies, the identified ciliary proteins may represent potential molecular targets for the development of new treatments.

Research perspectives

Mutation profile of ciliary genes can be analyzed directly from archived FFPE tissues of ADPKD by NGS. The first step, it is necessary to verify the results on a larger number of samples and matched tissues controls. Further research should be focused on the functional analysis of identified genetic variants. Consequently, it will be necessary to determine whether the inactivation of

these genes will lead to a change in the structure of renal cilia and affect the development or progression of ADPKD.

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P- Reviewer: Vega J **S- Editor**: Cui LJ **L- Editor**: A
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