同研究)のサンプルを用いた。本研究は京都大学大学院医学研究科及び大阪市立総合医療センターの医の倫理委員会の承認を受けており、文書による同意を示した症例のみを解析した。

C. 研究結果

糖尿病性腎症、肥満関連腎症、微小変化群、糸球体病変軽微の 4 群の症例を含むヒト腎生検組織での検討により、MRP8 陽性細胞は尿細管間質領域よりも糸球体内に優位に認められ、多いほうから糖尿病性腎症、肥満関連腎症、微小変化群、糸球体病変軽微の順であった。単変量解析では、糸球体内MRP8陽性細胞数ならびにMRP8陽性尿細管間質面積は、両者ともに収縮期血圧、蛋白尿、血清クレアチニン値、糸球体硬化、尿細管間質線維化と正相関していた。また腎生検 1 年後の蛋白尿を規定する独立した因子を多変量解析にて検討すると、糸球体内MRP8陽性細胞数(β=0.59, P<0.001)、蛋白尿(β=0.37, P<0.005)、収縮期血圧(β=0.21, P<0.05)の 3 つが選出された。とくに糸球体内 MRP8 陽性細胞数は、単変量解析では腎生検時の蛋白尿(β=0.78)よりもむしろ 1 年後の蛋白尿(β=0.87)と強い相関を示し、治療抵抗性の蛋白尿の指標となると考えられた。

D. 考察

糖尿病性腎症の腎生検組織で糸球体内の CD68 陽性マクロファージおよび尿細管間質領域の萎縮尿細管に MRP8 は強く発現していた。肥満関連腎症における MRP8 発現は糸球体病変軽微・微小変化群よりも強く、糖尿病性腎症よりも弱かった。横断的検討の多変量解析では、尿細管間質 MRP8 陽性面積は蛋白尿と尿細管間質線維化と相関を示し、糸球体内 MRP8 陽性細胞数は尿細管間質線維化と(微小変化群を除いた症例の)蛋白尿と相関を示した。縦断的検討の多変量解析では、腎生検 1 年後の蛋白尿は糸球体内 MRP8 陽性細胞数・生検時蛋白尿・血圧と関連していた。微小変化群では生検時蛋白尿が多いが、糸球体内の MRP8 発現は少なく、1 年後の蛋白尿も少なかったため、全体で評価すると、糸球体内 MRP8 陽性細胞数は、生検時よりもむしろ 1 年後の蛋白尿と非常に強い相関を示すと考えられた。

E. 結論

糸球体内 MRP8 の発現は糖尿病性腎症を含む慢性腎臓病において重症化のマーカーとなる可能性が考えられた。

F. 健康危険情報

特記事項なし

G. 研究発表

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H. 知的所有権の出願・取得状況

特記事項なし

研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表

雑誌 （謝辞があるものを以下に記載する）

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SPECIAL ARTICLE

A new classification of Diabetic Nephropathy 2014: a report from Joint Committee on Diabetic Nephropathy

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Abstract The Joint Committee on Diabetic Nephropathy has revised its Classification of Diabetic Nephropathy (Classification of Diabetic Nephropathy 2014) in line with the widespread use of key concepts such as the estimated glomerular filtration rate (eGFR) and chronic kidney disease. In revising the Classification, the Committee carefully evaluated, as relevant to current revision, the report of a study conducted by the Research Group of Diabetic Nephropathy, Ministry of Health, Labour and Welfare of Japan. Major revisions to the Classification are summarized as follows: (1) eGFR is substituted for GFR in the Classification; (2) the subdivisions A and B in stage 3 (overt nephropathy) have been reintegrated; (3) stage 4 (kidney failure) has been redefined as a GFR less than 30 mL/min/1.73 m^2, regardless of the extent of albuminuria; and (4) stress has been placed on the differential diagnosis of diabetic nephropathy versus non-diabetic kidney disease as being crucial in all stages of diabetic nephropathy.

Keywords Diabetic nephropathy · Chronic kidney disease (CKD) · Albuminuria · Proteinuria · Glomerular filtration rate (GFR)

Introduction

Diabetic nephropathy became the leading cause of chronic dialysis in 1998. Since then, the incidence of this condition has increased with only a recent plateau. However, diabetic nephropathy continues to account for a large proportion of all cases of chronic kidney disease (CKD) and remains by far the most common underlying cause of chronic dialysis among all kidney diseases [5], consequently leading to the escalation of healthcare costs, thus representing a compelling medico-social issue of interest.

The Classification of Diabetic Nephropathy (hereafter "Classification") developed earlier by the Research Group

Japan Diabetes Society, Japanese Society of Nephrology, Japanese Society for Dialysis Therapy, and Japan Society of Metabolism and Clinical Nutrition established the Joint Committee on Diabetic Nephropathy, which published the revised Classification of Diabetic Nephropathy 2014 in Japanese [1–4]. This is the English version of that revision.

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of Diabetic Nephropathy at the Ministry of Health, Labour and Welfare (MHLW) [6] and later revised by the Joint Committee on Diabetic Nephropathy (hereafter "Committee") [7] is widely used in Japan. However, as the concept of CKD was proposed, followed by the classification of CKD stages [8], it became clear that there exists a subpopulation of patients with discrepant classifications of diabetic nephropathy and CKD. This is thought to be due to the fact that diabetic nephropathy is primarily classified according to the extent of albuminuria in addition to the glomerular filtration rate (GFR) (i.e., creatinine clearance [CCr]), whereas CKD is primarily classified based on the estimated GFR [estimated GFR (eGFR)]. Meanwhile, eGFR has become increasingly used to assess GFR, and a new classification of CKD was developed in 2012 [9]. Against this background, the Committee therefore discussed issues of interest in depth and sought to develop a revision of the Classification.

Development of the 2014 Classification (Revised Classification) (see Table 1)

Prior to revising the Classification, as part of a MHLW-subsidized project on kidney disease, entitled "Diabetic Nephropathy Research, from the Ministry of Health, Labour and Welfare of Japan", a "historical cohort study" was conducted by the Research Group of Diabetic Nephropathy, MHLW, involving a total of 4,355 subjects with type 2 diabetes from 10 participating healthcare facilities with the aim of evaluating renal events (i.e., a decrease in eGFR to half the baseline level and/or the need for dialysis), cardiovascular events and all-cause mortality [10, 11]. Summarized below are the major findings of this study (for detailed information, please access the MHLW website http://www.mhlw.go.jp/ or refer to the literature cited above).

1. Renal and cardiovascular events and all-cause mortality were significantly increased in the subjects with micro- or macroalbuminuria compared to that observed in the subjects with normoalbuminuria.
2. In those with renal impairment (defined as a GFR less than 60 mL/min/1.73 m^2):
   a. The risk of renal events increased in association with the onset of microalbuminuria and further increased with the onset of macroalbuminuria in the subjects;
   b. The risk of cardiovascular events was increased in those with micro-/macroalbuminuria; and
   c. All-cause mortality was increased in the subjects with macroalbuminuria as well as those with normoalbuminuria and microalbuminuria who exhibited a GFR of less than 30 mL/min/1.73 m^2.

While that study was not a true prospective study and involved only a limited number of facilities and patients from a population known to be less prone to cardiovascular

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events than those in Western countries, the findings provide important insight into the prognosis of diabetic nephropathy in Japanese patients. Therefore, in seeking to revise the Classification, the Committee gave due consideration to the above findings. At the same time, the following considerations were also taken into account.

1. The bulk of evidence for the classification of diabetic nephropathy comes from randomized controlled studies enrolling patients with diabetic nephropathy as defined based on the extent of albuminuria, and very little evidence is available for diabetic nephropathy as defined based on GFR.
2. The current "Medical Service Fee Schedule for Guidance on Preventing Diabetes-Associated Dialysis" was developed with the Classification in mind.
3. The "Guidelines for Clinical Efficacy Evaluation of Pharmacological Agents for Diabetic Nephropathy (Draft)" currently in use were developed with the Classification in mind.

Therefore, after giving due consideration to all of these issues during the course of several sessions, the Committee decided to leave the Classification essentially unchanged for now (Table 1), while showing how it may be aligned with the widespread CKD classification based on GFR (eGFR) ("see Appendix"). The former is not, however, presented as a heat map, due to the limitations of the study referred to above, which involved a small number of patients with diabetic nephropathy and included no dialysis patients, providing the basis for this revision. Again, as all kidney diseases affecting patients with diabetes are covered in the Classification, the Committee called for attention with notes included which were required, in order to highlight the importance of the differential diagnosis between diabetic nephropathy and non-diabetic kidney disease in all stages. The differential diagnosis calls for collaboration with nephrologists; such collaboration is not limited to cases requiring a renal biopsy. Furthermore, given that the disease may not always progress in some patients, numerous notes were included in the table in order to call attention to these cases. Additionally, in view of the potential need to use multiple anti-diabetic drugs over time, "Key Precautions in View of Drug Use" are included below the table. The major revisions to the Classification are summarized below:

1. eGFR is now substituted for GFR in the Classification.

Table 1 Classification of Diabetic Nephropathy 2014

Stage	Urinary albumin (mg/g Cr) or urinary protein (g/g Cr)	GFR (eGFR) (mL/min/1.73 m^2)
Stage 1 (pre-nephropathy)	Normoalbuminuria (< 30)	≥30[a]
Stage 2 (incipient nephropathy)	Microalbuminuria (30–299)[b]	≥30
Stage 3 (overt nephropathy)	Macroalbuminuria (≥ 300) or Persistent proteinuria (≥ 0.5)	≥30[c]
Stage 4 (kidney failure)	Any albuminuria/proteinuria status[d]	<30
Stage 5 (dialysis therapy)	Any status on continued dialysis therapy	

Diabetic nephropathy does not always progress from one stage to the next. The revised classification takes into account findings on the prognosis of type 2 diabetic patients from a "historical cohort study" conducted as part of the MHLW-subsidized Project on Kidney Disease, entitled "Diabetic Nephropathy Research, from the Ministry of Health, Labour and Welfare of Japan" [10, 11]

[a] While a GFR of less than 60 mL/min/1.73 m^2 is consistent with the diagnosis of CKD, underlying causes other than diabetic nephropathy may be involved in patients with a GFR below 60 mL/min/1.73 m^2 thus calling for the differential diagnosis between diabetic nephropathy and any other potential non-diabetic kidney diseases

[b] Patients with microalbuminuria are to be diagnosed as incipient nephropathy after the differential diagnosis based on the criteria for an early diagnosis of diabetic nephropathy

[c] Precautions are required in patients with macroalbuminuria, in whom renal events (e.g., a decrease in eGFR to half its baseline value, the need for dialysis) have been shown to increase as the GFR decreases below 60 mL/min/1.73 m^2

[d] All patients with a GFR of less than 30 mL/min/1.73 m^2 are classified as exhibiting kidney failure, regardless of their urinary albumin/protein values. However, in those with normoalbuminuria and microalbuminuria, the differential diagnosis is required between diabetic nephropathy and any other potential non-diabetic kidney diseases

Key Precautions in View of Drug Use: This table is intended, first and foremost, as a classification of diabetic nephropathy and not as a guide to drug use. All drugs, including anti-diabetic drugs, particularly renally metabolized agents, are to be used in accordance with their prescribing information, with due consideration to relevant factors such as GFR in each patient

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2. The stages used in the Classification have been simplified to include normoalbuminuria, microalbuminuria, macroalbuminuria and kidney failure.
3. The division between A and B (early versus late macroalbuminuria) in stage 3 has been abandoned and A and B have been reintegrated, due to the paucity of evidence for proteinuria of 1 g/day as the threshold for dividing the stage.
4. Kidney failure has been redefined in all cases as a GFR less than 30 mL/min/1.73 m^2, which represents the threshold value for kidney failure obtained by quantifying the existing definition of kidney failure in the Classification based on the Classification of the Japanese Society of Nephrology (JSN) [12] with all other pre-kidney failure conditions redefined as a GFR of 30 mL/min/1.73 m^2 or greater.
5. Qualifying or illustrating phases in parentheses, such as "e.g., incipient nephropathy", have been retained throughout the Classification, as they have become common currency in the field, although their removal from the Classification was suggested during the process of revision.
6. Stress is now placed on the differential diagnosis of diabetic nephropathy versus non-diabetic kidney disease as being crucial in all stages of diabetic nephropathy.

Of note, the American Diabetes Association (ADA) proposed in its Clinical Practice Recommendations 2013 that all cases of albuminuria of 30 μg/mg Cr (=mg/g Cr) be defined as "increased urinary albumin excretion", thus abandoning the division between micro- and macroalbuminuria [13]. Again, while this concept was retained in the Clinical Practice Recommendations 2014, the ADA further proposed that microalbuminuria and macroalbuminuria be redefined as persistent albuminuria of 30–299 mg/24 h and ≥300 mg/24 h, respectively [14]. While this change may result in the terms micro- and macroalbuminuria ceasing to be common currency in the clinical setting in the US, to avoid confusion, the Committee has chosen not to follow suit and rather err on the side of caution, thereby retaining these terms in the Classification, given that they are less likely to no longer be used in scientific publications and are expected to remain common currency in Japan.

Last but not least, with a number of multicenter prospective studies currently underway, including the Japan Diabetes Complication and Prevention prospective (JDCP) study, JSN registries, Japan Diabetes Clinical Data Management (JDDM) studies and Japan Diabetes Optimal Integrated Treatment for 3 Major Risk Factors of Cardiovascular Diseases (J-DOIT3) randomized study, the Committee also plans to further revise the Classification in a timely fashion as required, as relevant evidence becomes available from these and other studies.

Conclusions

In order to resolve the discrepancy between the existing Classification of Diabetic Nephropathy and the current Classification of CKD stages, the Joint Committee on Diabetic Nephropathy revised its Classification of Diabetic Nephropathy. The new classification has already been uploaded onto the website of each member society represented on the Joint Committee as of January 10, 2014. Again, in view of further revisions in the years to come, the Joint Committee has termed the revised classification as the "Classification of Diabetic Nephropathy 2014."

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Conflict of interest Masakazu Haneda has received speaker honoraria from pharmaceutical companies Boehringer Ingelheim GmbH, Mitsubishi Tanabe Pharma Corporation, Novo Nordisk Pharma Ltd., Daiichi-Sankyo Co., Ltd., Taisho Pharmaceutical Co., Ltd., Sanofi K.K., Merck Sharp & Dohme, Astellas Pharma Inc., Kyowa Hakko Kirin Co., Ltd., Kowa Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Novartis Pharma K.K., scholarship grants from Astellas Pharma Inc., Daiichi-Sankyo Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical Co., Ltd., Novo Nordisk Pharma Ltd., Merck Sharp & Dohme, Boehringer Ingelheim GmbH, and Eli Lilly and Company; Daisuke Koya has received speaker honoraria from pharmaceutical companies Mitsubishi Tanabe Pharma Corporation, Boehringer Ingelheim GmbH, and Eli Lilly and Company, research grants from Mitsubishi Tanabe Pharma Corporation, Boehringer Ingelheim GmbH, Japan Tabacco Inc., Eli Lilly and Company, and Ono Pharmaceutical Co., Ltd.; Tetsuya Babazono has received speaker honoraria from pharmaceutical company Merck Sharp & Dohme; Tatsumi Moriya has received travel expenses from pharmaceutical companies Astellas Pharma Inc., Takeda Pharmaceutical Co., Ltd., Novo Nordisk Pharma Ltd., and Daiichi-Sankyo Co., Ltd.; Hirofumi Makino has received speaker honoraria from pharmaceutical companies Teijin Pharma Limited, Chugai Pharmaceutical Co., Ltd., AbbVie GK, Astellas Pharma Inc., Boehringer Ingelheim GmbH, Daiichi-Sankyo Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Merck Sharp & Dohme, Novartis Pharma K.K., Pfizer Japan Inc., Takeda Pharmaceutical Co., and Mitsubishi Tanabe Pharma Corporation, research grants from Project for accelerating Practice and Research on Community Medicine in Okayama Prefecture, scholarship grants from Astellas Pharma Inc., Daiichi-Sankyo Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Merck Sharp & Dohme, Takeda Pharmaceutical Co., Ltd., Mochida Pharmaceutical Co., Ltd., Novo Nordisk Pharma Ltd., and Mitsubishi Tanabe Pharma Corporation; Kenjiro Kimura has received research grants from pharmaceutical companies Otsuka Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., Teijin Pharma Limited, Boehringer Ingelheim GmbH, Baxter International Inc., and Sekisui Medical Co., Ltd.; Takashi Wada has received speaker honoraria from pharmaceutical company Daiichi-Sankyo Co., Ltd., scholarship grants from Chugai pharmaceutical Co., Ltd.; Susumu Ogawa has received speaker honoraria from pharmaceutical companies Daiichi-Sankyo Co., Ltd., Eli Lilly and Company, and Novo Nordisk

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Human rights statement and Informed consent This article does not contain any studies with human or animal subjects performed by the any of the authors.

Appendix

Relationship between the 2014 categories for diabetic nephropathy stages and the CKD severity categories

	Albuminuria category	A1	A2	A3
	Quantitative urinary albumin estimation Urinary albumin/Cr ratio (mg/g Cr) (quantitative urinary protein estimation) (urinary protein/Cr ratio (g/g Cr)	Normoalbuminuria < 30	Microalbuminuria 30-299	Macroalbuminuria ≥300 (or increased proteinuria) (≥0.50)
GFR category (mL/min/1.73 m^2)	≥90 60-89 45-59 30-44	Stage 1 (pre-nephropathy)	Stage 2 (incipient nephropathy)	Stage 3 (overt nephropathy)
	15-29 < 15	Stage 4 (kidney failure)		
	(dialysis therapy)	Stage 5 (dialysis therapy)		

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Pro-inflammatory/Th1 gene expression shift in high glucose stimulated mesangial cells and tubular epithelial cells

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ABSTRACT

Diabetic nephropathy (DN) is a major cause of end stage kidney disease and a strong risk factor for cardiovascular diseases. Growing data show chronic inflammation plays an important role for the progression of DN. As for the immune cells, anti-inflammatory leukocytes as well as pro-inflammatory leukocytes play an important role in DN. In addition to leukocytes, renal resident cells contribute to the inflammatory process of DN. However, precise functions, phenotypes and immune balance of renal resident cells remain to be explored. Therefore, we hypothesized that the aberrant immune balance of renal resident cells contributes to the pathogenesis of DN. To explore this possibility, we performed genome-wide transcriptome profiling in mesangial cells and tubular epithelial cells (TECs), which were stimulated by high glucose (HG) and detected the expression of inflammation associated genes. HG increased the mRNA expression of oxidative stress, inflammasome and mammalian target of rapamycin (mTOR) related genes in mesangial cells. Pro-inflammatory/Th1 gene expression was upregulated, but Th2 related gene expression was downregulated in mesangial cells. In TECs, HG stimulation increased pro-inflammatory/Th1/Th2 gene expression. Phosphorylation of signaling proteins shifted towards pro-inflammatory phenotype with suppressed phosphorylation of Th2 related signaling in TECs. The data taken together indicate that HG shifts the immune balance toward pro-inflammatory/Th1 phenotype in mesangial cells and TECs, which might initiate and/or prolong inflammation, thereby resulting in diabetic nephropathy.

1. Introduction

The number of patients with end stage kidney diseases (ESKD) is increasing worldwide. Especially, diabetic nephropathy (DN) is one of the major causes of ESKD in developed countries. Not only the high incidence of ESKD, but also the high mortality from cardiovascular diseases has been observed in patients with DN [1]. Therefore, identifying molecular mechanisms that mediate the progression of DN is an important issue to uncover novel therapeutic targets.

Growing evidence suggests that chronic inflammation plays key roles for the progression of DN [2–4]. Recent studies revealed that macrophages (Mϕ) and T cells contribute to the pathogenesis of DN in both rodent and human models. Activated Mϕ and T cells induce and aggravate the kidney injury, resulting in chronic kidney failure. Recently, Mϕ are categorized as inflammatory (M1) and anti-inflammatory (M2) phenotype according to the cytokine profile and cell surface marker [5]. M2 Mϕ as well as M1 Mϕ has been reported to contribute to the pathogenesis of DN [6–8]. $CD4^+$ T cell populations are also broadly and simplistically divided into 4 types, Th1, Th2, Th17 and regulatory T cells based on the function [9]. Each phenotype of Th cell has been reported as a key player for the progression of DN [10–12]. These data suggest that the immune balance of inflammatory cells affect the prognosis of DN.

Not only accumulated leukocytes, but also renal resident cells orchestrate in the inflammatory processes in DN. Activated renal resident cells secret various cytokines/chemokines [13,14]. However, precise functions, phenotypes and immune balance of renal resident cells remain to be explored in DN. Therefore, we hypothesized that the aberrant immune balance of renal resident cells

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may contribute to the pathogenesis of DM. To explore this hypothesis, we performed genome-wide transcriptome profiling in mesangial cells and tubular epithelial cells (TECs), which were stimulated by high glucose (HG), and identified the expression of inflammatory associated genes.

2. Materials and methods

2.1. Cell culture

Normal human mesangial cells (Lonza, Basel, Switzerland) were cultured in mesangial culture kit (Lonza). Human immortalized TECs, HK-2 (ATCC, Manassas, VA) was cultured in 10% FBS DMEM (Life technologies, Tokyo) with 100 μg/ml streptomycin and 100 U/ml penicillin. All the cells were grown in a humidified atmosphere (5% CO^2/95% air) at 37 °C and were seeded onto six-well cell culture cluster (Corning Incorporated, Corning, NY). The cells were stimulated with 30 mM glucose (Wako, Tokyo) for 24 h for hyperglycemic stimulation. For osmotic control, the cells were cultured with 30 mM mannitol (Wako) for 24 h.

2.2. SAGE analysis using Ion Torrent PGM™

Gene expression analysis was performed by SAGE method and NGS. Briefly, RNA was isolated using mRNA Isolation kit (Sigma–Aldrich, St. louis, MO). NGS data from the PGM™ was generated from 1 μg of total RNA isolated from cell line. SAGE libraries were constructed using the SOLiD SAGE™ kit from Life Technologies (Life Technologies) according to manufacturer's protocol. DNA was recovered from the agarose gel using PureLink Gel Extraction kit (Life Technologies). DNA fragments of SAGE construct were analyzed on the Agilent Bioanalyzer using the High Sensitivity Kit (Agilent, Santa Clara, CA). Template preparation and emulsion PCR, and Ion Sphere Particles (ISP) enrichment was done using the Ion Xpress Template kit (Life Technologies) according to the manufacturer's instructions. The quality of the resultant ISPs was assessed using Qubit 2.0 Fluorometer (Life Technologies), and were loaded and sequenced on a 318 chip (Life Technologies). The complete data sets from these experiments have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession number GSE52734).

2.3. Mapping from NGS data

For each of the samples, the PGM™ raw reads were aligned against the human refseq genes (UCSC, http/hgdownload.cse.ucsc.edu/) using BWA 0.6.2 which uses the 25_1 mapping parameter. We generated unique gene counts by excluding reads that mapped to contigs of more than one gene. Reads mapping to several contigs within an isogroup were only counted once.

2.4. Antibodies

The following antibodies were used for flow cytometric analysis; Phycoerythrin (PE) conjugated anti-phosphorylated p38 (612565; BD Biosciences, San Jose, CA), PE-conjugated anti-phosphorylated STAT1 (562674; BD Biosciences), PE-conjugated anti-phosphorylated STAT3 (558557; BD Biosciences), PE-conjugated anti-phosphorylated STAT5 (61257; BD Biosciences), PE-conjugated anti-phosphorylated STAT6 (612701; BD Biosciences).

2.5. Flow cytometric analysis

To detect phosphorylated intracellular signaling, cells were fixed and permeabilized using BD Phosflow™ in accordance with the manufacturer's protocol (BD Biosciences). Then, the cells were stained with antibody. We collected 10,000 cultured cells using FACSCalibur (BD Biosciences) and analyzed data using FlowJo software 9.3 (Tree Star, Palo Alto, CA).

2.6. Reconstitution of signal transduction pathway

To identify the signal transduction pathway, each gene was reconstituted according to Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.genome.jp/kegg/) database.

2.7. Statistics

Statistical analysis was performed as described before [15]. Data represent the expression ratio (HG stimulated/osmotic control).

3. Result

3.1. High glucose (HG) increased the mRNA expression of oxidative stress, inflammasome and mammalian target of rapamycin (mTOR) related genes

Oxidative stress [16,17] has been regarded as intracellular mediator in HG induced inflammation. Thus, we examined the mRNA expression of oxidative stress associated genes. Among them, reactive oxygen species modulater 1 (ROMO) and protein kinase C (PKC) alpha (PRKCA) mRNA were increased in HG stimulated mesangial cells (Fig. 1). Recent study revealed that PKC signaling is closely related to mTOR signaling [18,19]. Therefore, we analyzed mTOR signaling related genes. Interestingly, the relative gene expression of mTOR and its downstream signaling, translation initiation factor 4B (EIF4B) were upregulated. Moreover, thioredoxin interacting protein (TXNIP) mRNA, which has the potential to activate the NOD-like receptor family, pyrin domain containing (NLRP) 3 inflammasome [20], was also increased in HG stimulated mesangial cell. In addition, transcription factor, activated protein (AP)-1 (JUN, FOSL2), C–C chemokine ligand (CCL) 2 and tumor necrosis factor (TNF)-α mRNA were also increased higher in HG stimulated mesangial cells as compared to osmotic control.

3.2. HG stimulated mesangial cells were skewed toward an inflammatory phenotype

As HG increased the mRNA expression levels of inflammatory signal mediators, we look into whether HG stimulation shifted the cell phenotype toward an inflammatory population. Inflammatory cytokines/chemokines and their receptors, such as CCL2, TNF-α and interleukin(IL)-1 receptor mRNA expression were increased by the HG stimulation in mesangial cells. Moreover, mitogen activated protein kinase (MAPK) mRNA expression was also increased in HG stimulated mesangial cells. In contrast, Th2 related cytokine receptors and intracellular signaling molecules showed the reduced mRNA expression levels (Table 1). Taken together, HG stimulation increased mRNA expression of the pro-inflammatory molecules and decreased Th2 related molecules in mesangial cells.

3.3. Increased mRNA expression of inflammatory cytokines/chemokines and Th2 type cytokine receptors in HG stimulated TECs

Next, we examined the functional phenotype of HG stimulated TECs. Stimulated TECs showed the increased mRMA levels of inflammatory cytokines/chemokines and their receptors, such as TNF-α, IL-1 receptor, IL-18, IL-12 and IL-6. In addition, mRNA expression of Th2 related cytokine receptors, IL-10 receptor and IL-13 receptor, also increased via HG stimulation (Table. 2).

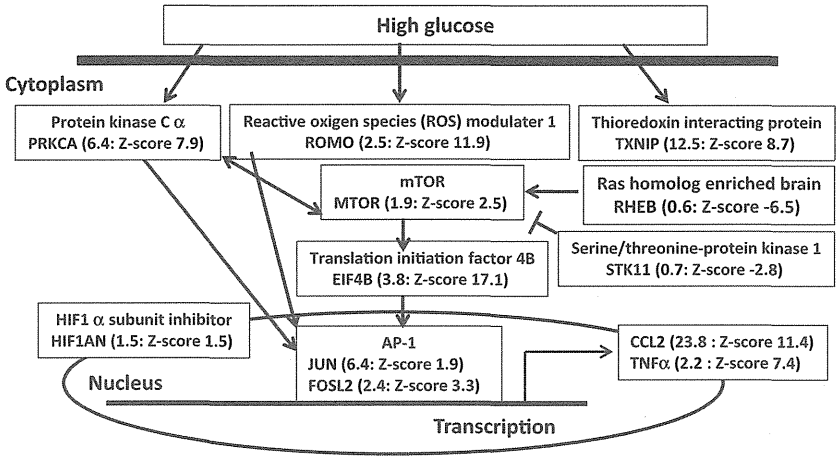

Fig. 1. High glucose (HG) increased the mRNA expression of oxidative stress, inflammasome and mammalian target of rapamycin (mTOR) related genes. HG increased the mRNA expression of reactive oxygen species modulater 1 (ROMO), protein kinase C alpha (PRKCA) and thioredoxin interacting protein (TXNIP). Moreover, gene expression of mTOR and its downstream signaling, translation initiation factor 4B (EIF4B) were also upregulated. Data represents the ratio of gene expression (HG stimulated mesangial cells/mannitol treated mesangial cells).

Table 1
HG stimulated mesangial cells were skewed toward an inflammatory phenotype.

	Increased genes ↑ (High glucose/mannitol control)		Decreased genes ↓ (High glucose/mannitol control)	
Pro-inflammatory	CCL2 (23.8)	MAPK1 (1.6)		MAPK7 (0.7)
	TNFα (2.2)	MAPK3 (1.1)		
	IL-1R (2.0)	MAPK6 (1.7)		
Th1		STAT1 (1.3)	IFN-γR (0.5)	
Th2			IL-4R (0.5)	STAT5 (0.5)
			IL-10Rβ (0.9)	STAT6 (0.5)
			IL-13Rβ (0.8)	
Th17	IL-6ST (1.3)		IL-6 (0.5)	STAT3 (0.3)
TGF		SMAD1 (1.3)		SMAD3 (0.4)
		SMAD2 (1.7)		

Abbreviations are CCL: CC chemokine ligand, TNF: tumor necrosis factor, MAPK: mitogen activated protein kinase, STAT: signal transducer and activator of transcription, IFN: interferon, IL: interleukin, SMAD: Sma and Mad related family.

Table 2
Increased mRNA expression of inflammatory cytokine/chemokine and Th2 type cytokine receptor in HG stimulated TECs.

	Increased genes ↑ (High glucose/mannitol control)		Decreased genes ↓ (High glucose/mannitol control)	
Pro-inflammatory	TNFα (1.5)	MAPK1 (1.3)		MAPK7 (0.6)
	IL-1R (3.0)			
Th1	IL-18 (1.8)	STAT1 (2.8)	IFN-γR (0.8)	
	IL-12 (2.0)			
Th2	IL-10Rβ (1.7)			
	IL-13Rα (2.0)			
Th17	IL-6 (3.6)			
TGF				TGF-β1 (0.8)

Abbreviations are TNF: tumor necrosis factor, MAPK: mitogen activated protein kinase, STAT: signal transducer and activator of transcription, IFN: interferon, IL: interleukin, TGF: transforming growth factor.

3.4. Phosphorylation of intracellular signaling molecules were increased in HG stimulated mesangial cells and TECs

Intracellular signaling has crucial roles in cytokines/chemokines- mediated cellular reactions, such as proliferation, differentiation and activation. The phosphorylation of signaling proteins is essential for the activation of the signal transduction pathways. Therefore, we examined the phosphorylation of signaling proteins in HG stimulated mesangial cells and TECs. Phosphorylated form of p38 MAPK and signal transducer and activator of transcription (STAT) 3, which are related to pro-inflammatory/Th1 signaling [21] were significantly higher in 24 h HG stimulated mesangial cells and TECs. Conversely, phosphorylated forms of STAT5 and STAT6, which are associated with Th2 type signaling transductions [21], were significantly lower in 24 h HG stimulated TECs (Fig. 2). These data suggest the shift of the immune balance toward pro-inflammatory phenotype in mesangial cells and TECs stimulated with HG.

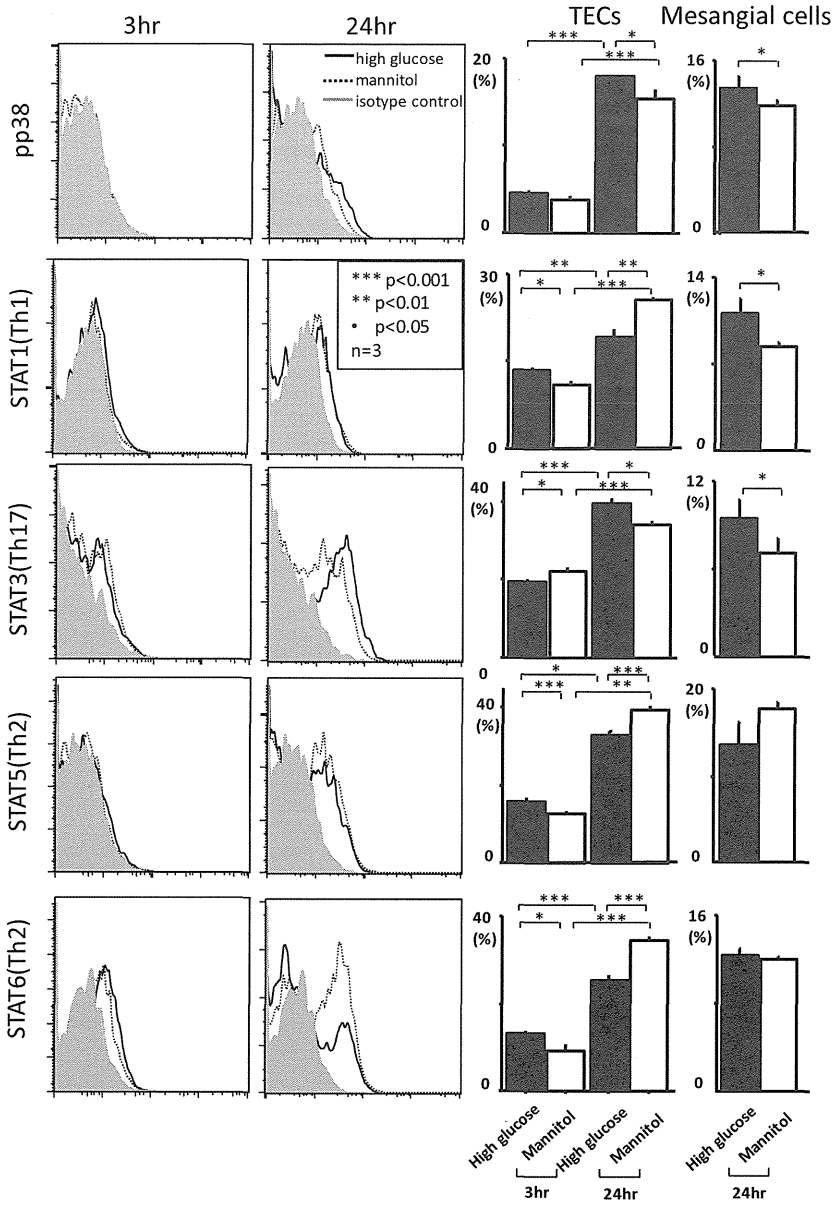

Fig. 2. Pro-inflammatory/Th17 related phosphorylated intracellular signaling were increased in HG stimulated mesangial cells and TECs. Phosphorylated p38 MAPK and STAT3 was significantly higher in 24 h HG stimulated mesangial cells and TECs. Conversely, phosphorylated STAT5 and STAT6 were significantly lower in 24 h HG stimulated TECs. Data represents means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

4. Discussion

We now report that HG stimulation increases pro-inflammatory/Th1 gene expression, but decreases Th2 related gene expression in mesangial cells. In TECs, HG stimulation increased pro-inflammatory/Th1/Th2 gene expression. Phosphorylation of signaling proteins shifted towards pro-inflammatory phenotype with suppressed phosphorylation of Th2 related signaling proteins in mesangial cells and TECs. The data taken together indicate that HG shifts the immune balance toward pro-inflammaotry/Th1 phenotype in mesangial cells and TECs, which might initiate and/or prolong inflammation, thereby resulting in diabetic nephropathy.

Growing evidence suggests that chronic inflammation plays important roles in the progression of DN [2–4]. Not only the leukocytes, but also renal resident cells, including mesangial cells and TECs participate in the pathogenesis of DN. HG stimulation induced the gene expression of PKC and ROS related proteins in mesangial cells. PKC and ROS related proteins have been reported as the key signaling for HG induced mesangial cell damage [16,17]. Therefore, our data suggest HG stimulation activated intracellular signaling pathway in mesangial cells. Interestingly, the relative gene expression of mTOR and its downstream signaling, translation initiation factor 4B (EIF4B) were upregulated. Supporting this notion, the recent study revealed that PKC signaling is closely related to mTOR signaling [18,19]. Moreover, mTOR expression has been reported in mesangial cells and podocytes in DN [22,23]. In addition, thioredoxin interacting protein (TXNIP) gene expression was also increased in HG stimulated mesangial cells. Lerner et al. reported that increased TXNIP activate NLRP 3 inflammasome, causing procaspase-1 cleavage and IL-1β secretion [20]. Another group also reported the involvement of inflammasome in DN [24]. Thus, our data suggest HG stimulation activate mTOR signaling and

inflammasome in cultured mesangial cells. However, more precise analysis are needed to confirm if these signaling pathway is really involved in DN.

Recently, Mϕ are categorized as inflammatory (M1) and anti-inflammatory (M2) phenotype according to the cytokine profile and cell surface markers [5]. $CD4^+$ T cell populations are also divided into 4 types, Th1, Th2, Th17 and regulatory T cells based on the function [9]. Orchestration of inflammation by pro-inflammatory cells with anti-inflammatory cells has an impact on the process of progressive kidney diseases including DN. In this study, mesangial cells and TECs displayed pro-inflammatory phenotype with the reduction of Th2 related genes via HG stimulation. In support of our findings, Min et al. reported that HG increases TNF-α and IL-6 secretion in mesangial cells [25]. Also tubular epithelial cells have been reported as an important source for cytokines/chemokines in diabetes [14]. Moreover, suppression of JAK/STAT signaling decreased the expression of pro-inflammatory cytokines/chemokines, resulting in improved kidney injury in a rat diabetes model [26]. In addition, we have reported that repairing TECs from hypoxia injury releases mediator that skews M1 Mϕ toward M2 phenotype [27]. The data taken together indicate that renal resident cells such as mesangial cells and TECs orchestrate in the inflammatory processes with changing the immune balance.

As for the intracellular signaling pathway, phosphorylation plays critical roles in signal transduction. Thus, we analyzed the phosphorylation of each molecule. HG stimulation for 24 h increased the phosphorylation of p38 and STAT3. P38 MAPK is a key signaling for inflammatory cytokines/chemokines, thereby contributing to the progression of inflammatory kidney diseases [28–31], including DN [8]. STAT3 pathway is required for IL-23/IL-17 signaling, thereby leading helper T cell to Th17 axis [21]. Supporting our notion, STAT3 expression has been reported in mesangial cell [32], mediating cell proliferation and activation. Moreover, Ranganathan et al. reported the increased collagen expression via STAT3 activation in TECs [33]. However, the association STAT3 and Th17 axis is not clear in renal resident cells. Loverre et al. reported the IL-17 expression in TECs in renal transplant recipients with acute rejection, though STAT3 activation was not mentioned [34].

In contrast, STAT5 and STAT6 phosphorylation were decreased in HG stimulated TEC. IL-2/STAT5 and IL-4/STAT6 signalings promote Th2 polarization in CD4 helper T cell [21,35]. Interestingly, STAT6 signaling showed reno-protective potential in crescentic glomerulonephritis [36] and ischemia–reperfusion (I/R) injury [37]. Moreover, STAT5 signaling plays a central role in erythropoietin mediated tissue protection in I/R injury [38] and cisplatin induced acute kidney injury [39]. Therefore, the reduction of STAT6 and STAT5 signaling might suggest the blunted protective effect in HG stimulated TECs. However, the precise role of STAT5 and STAT6 signalings in DN remains to be investigated.

In conclusion, HG stimulation changes the immune balance toward inflammatory phenotype in mesangial cells and TECs, which might initiate and/or prolong inflammation, thereby resulting in DN. We anticipate that future studies elucidate the precise mechanisms of aberrant immune balance and thereby provide novel therapeutic approaches to DN.

Conflict of interest

The authors have declared that no conflict of interest exists.

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ORIGINAL ARTICLE

Impact of kidney function and urinary protein excretion on pulmonary function in Japanese patients with chronic kidney disease

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Abstract
Background Although the cardiorenal relationship in chronic kidney disease has been investigated, information about the lung–kidney relationship is limited. Here, we investigated the impact of kidney function and urinary protein excretion on pulmonary dysfunction.
Methods The data from pulmonary function tests and kidney function (estimated glomerular filtration rate [eGFR] and urinary protein) between 1 April 2005 and 30 June 2010 were selected from our laboratory database. Data were classified into 4 categories according to eGFR and proteinuria. Category 1, eGFR ≥60 ml/min/1.73 m^2 and urinary protein <0.3 g/gCr; category 2, eGFR <60 ml/min/1.73 m^2 and urinary protein <0.3 g/gCr; category 3, eGFR ≥60 ml/min/1.73 m^2 and urinary protein ≥0.3 g/gCr; and category 4, eGFR <60 ml/min/1.73 m^2 and urinary protein ≥0.3 g/gCr. Pulmonary function data were evaluated according to these 4 categories.
Results A total of 133 participants without major respiratory disease, abnormal computed tomography and smoking history were enrolled. Hemoglobin (Hb)-adjusted percentage carbon monoxide diffusing capacity (%DL_{CO}) in category 4 (46.2 ± 7.5) and category 2 (63.6 ± 17.8) were significantly lower than in category 1 (75.8 ± 18.9) ($P < 0.05$). In addition, Hb-adjusted %DL_{CO} was weakly correlated with eGFR in participants with urinary protein <0.3 g/gCr ($R = 0.30$, $P = 0.001$). Hb-adjusted %DL_{CO} was strongly correlated with eGFR in participants with urinary protein ≥0.3 g/gCr ($R = 0.81$, $P < 0.001$). Other pulmonary function test markers (percentage (%) vital capacity, % forced expiratory volume in one second (FEV1), FEV1/forced vital capacity, % total lung capacity, and % residual volume) were not significantly different between categories.
Conclusion This study suggests that decreased eGFR is associated with decreased %DL_{CO} in proteinuric patients.

Keywords Kidney function · Pulmonary function tests · Chronic kidney disease · Urinary protein · DL_{CO} (carbon monoxide diffusing capacity) · Diabetic nephropathy

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Background

The kidneys maintain whole-body homeostasis through regulation of body fluid, blood pressure, electrolytes and acid–base balance. Thus, the kidneys work in cooperation with the heart, liver, lungs and other organs. Various mechanisms participate in the organ-to-organ networks,

including humoral factors, and neuronal network systems. Although several reports indicate a relationship between the kidneys and the heart [1, 2], limited information is available regarding crosstalk between the kidneys and lungs in chronic kidney disease (CKD).

In contrast to CKD, acute kidney injury (AKI) often leads to acute lung injury. These associations between the kidneys and lungs [3–6] result in deterioration of the general condition of patients in critical care. Recent animal studies indicate that AKI increases inflammatory cytokine levels in the injured kidney and serum, and these cytokines may induce lung injury [7–9]. However, inflammatory cytokine production is also detected in the injured kidneys in CKD as well as in AKI [10]. Therefore, it is reasonable to speculate that CKD may affect pulmonary function.

The present study was performed to examine the associations between parameters of CKD (estimated glomeular filtration rate [eGFR] and urinary protein) and the results of pulmonary function tests (PFTs).

Method

Subjects

The data from PFTs and kidney function parameters (eGFR and urinary protein) were selected from our laboratory database between 1 April 2005 and 30 June 2010. PFTs were evaluated by international criteria [11]. Vital capacity (VC), forced vital capacity (FVC), functional residual capacity (FRC), and carbon monoxide diffusing capacity (DL_{CO}) were measured using the PFT system CHESTAC-9800 (Chest M.I., Inc., Tokyo, Japan). Participants were coached regarding standard forced expiratory manoeuvres. Three technically acceptable blows were recorded and the best values were used as their data.

We selected participants with normal spirometry (both %VC >80 % and forced expiratory volume in one second (FEV1)/FVC >70 %) to evaluate the association between renal function and pulmonary function. Participants with abnormal spirometry, major respiratory disease (asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, interstitial pneumonia, neuromuscular disease, scleroderma, pulmonary resection, and pneumonedema) and abnormal computed tomography (CT) were excluded. Moreover, participants with a history of smoking were excluded to remove the influence of smoking on kidney [12] and lung fucntion [13]. PFT markers (%VC, %FEV1, % residual volume [RV], % total lung capacity [TLC], and $\%DL_{CO}$) were adjusted for age, height, and sex using prediction formula.

This study was approved by the ethics committee of Kanazawa University Hospital (Approval No. 907) and was conducted in accordance with the Declaration of Helsinki.

Method of DLco

We measured DLco in accordance with the recommendations [14]. In short, once the mouthpiece is in place, four to five tidal volumes are recorded to determine a regular end-expiratory baseline. The DLco manoeuvre then begins with exhalation to RV. At RV the subject's mouthpiece is connected to a source of test gas and the subject inhales rapidly to TLC. The volume of test gas inhaled is V_I. DL_{CO} should be measured near TLC.

Participants' lung and kidney data function

eGFR (ml/min/1.73 m^2) was calculated using the prediction formula 194 × creatinine $(Cr)^{-1.094}$ × age $(year)^{-0.287}$ (multiplied by 0.739 for females) developed by the Japanese Society of Nephrology. Urinary protein (g/gCr) was evaluated by spot urine protein–creatinine ratio.

Pulmonary function was evaluated using PFT markers of restrictive ventilatory impairment (%VC, %RV, and %TLC), PFT markers of obstructive ventilatory impairment (%FEV1 and FEV1/FVC), and PFT markers of pulmonary diffusing capacity ($\%DL_{CO}$). To avoid the effects of physical features (age, sex and height), we used the predicted value of all PFT markers. Because predicted value is corrected by physical features, PFT markers are not influenced by physical features [13]. Furthermore, to avoid the effects of renal anaemia on CKD, $\%DL_{CO}$ was corrected by haemoglobin (Hb-adjusted $\%DL_{CO}$). The compensation formula of DL_{CO} for males is DL_{CO} × (10.22 + haemoglobin)/(1.7 × haemoglobin) [14, 15]. For females, it is DL_{CO} × (9.38 + haemoglobin)/(1.7 × haemoglobin).

Participants were classified into 4 categories according to eGFR and urinary protein—category 1, eGFR ≥60 ml/min/1.73 m^2 and urinary protein <0.3 g/gCr, category 2, eGFR <60 ml/min/1.73 m^2 and urinary protein <0.3 g/gCr; category 3, eGFR ≥60 ml/min/1.73 m^2 and urinary protein ≥0.3 g/gCr; and category 4, eGFR <60 ml/min/1.73 m^2 and urinary protein ≥0.3 g/gCr. Participants with CKD had an eGFR <60 ml/min/1.73 m^2 or/and U-PCR >0.3 g/gCr for at least 3 months. Pulmonary function was compared using these 4 categories.

Statistics

One-way analysis of variance (ANOVA) and Dunnett's post hoc test were used to compare PFT markers in category 1 to those in other categories. The chi-squared test was used for categorical parameters. Spearman's rank correlation test and multiple linear regression analysis were used to examine relationships between PFTs (Hb-adjusted $\%DL_{CO}$) and renal function (eGFR and urinary protein).

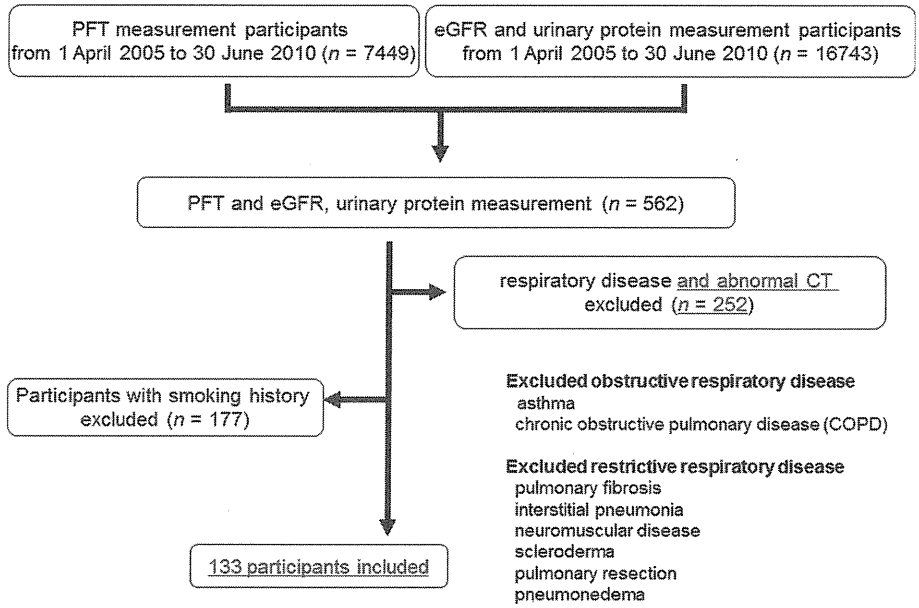

Fig. 1 Inclusion and exclusion criteria

Stata 12 (Stata Corp, College Station, TX, USA) was used for all statistical analyses. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

Participant selection and baseline characteristics

A total of 7,449 participants undergoing PFTs and 16,743 participants undergoing eGFR and urinary protein measurement were enrolled between 1 April 2005, and 30 June 2010 at Kanazawa University Hospital. A total of 562 participants underwent both PFTs and renal function tests. We excluded 252 participants with major respiratory disease and abnormal CT and 177 participants with smoking history. Thus, 133 participants were finally included in this study (Fig. 1).

Table 1 shows the characteristics of the subjects. Neither age, body mass index (BMI), Hb, brain natriuretic peptide (BNP) nor the presence of diabetes and hypertension were significantly different between categories. As we excluded participants with smoking history, females were predominant. The percentages of males in category 2 (2.3 %) and category 4 (0 %) were significantly lower than those in category 1 (32.4 %) and category 3 (22.2 %).

Changes in pulmonary function according to eGFR and urinary protein excretion

Table 2 shows a comparison between PFT parameters in each category. Hb-adjusted $\%DL_{CO}$ in category 4 (46.2 ± 7.5) and category 2 (63.6 ± 17.8) were significantly lower than in category 1 (75.8 ± 18.9) ($P < 0.05$). Other PFT markers (%VC, %FEV1, FEV1/FVC, %TLC, and %RV) were not significantly different between the categories.

Association between Hb-adjusted $\%DL_{CO}$ and eGFR and urinary protein excretion

Figure 2 shows the results of single regression analysis between Hb-adjusted $\%DL_{CO}$ and kidney function. Hb-adjusted $\%DL_{CO}$ was significantly positively correlated with eGFR ($R = 0.36$, $P < 0.001$, Fig. 2a), but was significantly negatively correlated with urinary protein ($R = -0.25$, $P = 0.005$, Fig. 2b).

Figure 3 shows the results of single regression analysis between Hb-adjusted $\%DL_{CO}$ and eGFR. Hb-adjusted $\%DL_{CO}$ was weakly correlated with eGFR in patients with urinary protein <0.3 g/gCr ($R = 0.30$, $P = 0.001$, Fig. 3a), but was strongly correlated with eGFR in patients with urinary protein ≥0.3 g/gCr ($R = 0.81$, $P < 0.001$, Fig. 3b).

To examine the precise relationships between kidney function and Hb-adjusted $\%DL_{CO}$, multiple linear regression analysis was conducted in all patients examined in this study. As shown in Table 3, eGFR was associated with Hb-adjusted $\%DL_{CO}$ ($P = 0.023$) after adjusting for physical features (age and gender), expiratory minute volume (VE), and the presence or absence of DM and Hypertension. Urinary protein tended to be associated with Hb-adjusted $\%DL_{CO}$ ($P = 0.095$) after adjusting for physical features (age and gender), VE, and the presence or absence of DM, and hypertension.

Discussion

This study was performed to examine the associations between kidney function or urinary protein excretion and