Association of chitotriosidase enzyme activity and genotype with the risk of nephropathy in type 2 diabetes

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ABSTRACT

Objective: The immune-inflammatory system has been implicated in the pathogenesis of diabetic nephropathy; however, many of the mechanisms involved remain unclear. Chitotriosidase enzyme is an active human chitinase and a major protein product of activated macrophages. Although playing an important role in innate and acquired immunity, chitotriosidase involvement in the development of diabetic nephropathy is unknown.

Design and methods: Chitotriosidase enzyme activity and the presence of the functional 24-bp duplication mutation of the chitotriosidase gene (CHIT1) were assessed in 262 Egyptian type 2 diabetic patients with and without nephropathy and 90 non-diabetic controls. In diabetic patients, multiple linear regression models were adapted to assess the association of chitotriosidase activity with two important measures of renal disease progression: urinary albumin/creatinine ratio and eGFR, while the association of the CHIT1 genotype with the incidence of nephropathy was evaluated by multiple logistic regression.

Results: In diabetic patients, chitotriosidase enzyme activity showed a statistically significant elevation as compared to controls and correlated positively with the progression of nephropathy. A significant association of chitotriosidase activity with both urinary albumin/creatinine ratio and eGFR was detected after adjusting for age, gender, duration of diabetes, body mass index, hypertension status, total cholesterol, triglycerides and HbA1c levels, P < 0.001. We also identified a protective association between the CHIT1 mutated genotype and diabetic nephropathy after adjusting for the same confounders (odds ratio: 0.517, 95% CI: 0.289–0.924, P = 0.026).

Conclusions: This study demonstrates for the first time that the immunomodulatory effects of chitotriosidase enzyme could be implicated in the development of nephropathy in type 2 diabetes.

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1. Introduction

Diabetic nephropathy is an important microvascular complication of diabetes mellitus and the most common cause of end stage renal disease (ESRD) [1]. About 20 to 40% of patients with type 1 or type 2 diabetes develop evidence of nephropathy and for type 2 less than 40% of those will progress to ESRD [2]. The development of diabetic nephropathy is a very complex process affected by age, ethnicity, duration of diabetes, glycemic control status, associated hypertension, life style factors and multiple genetic predispositions [3,4]. Many studies including in vitro cellular experiments, animal model experiments and epidemiological studies linked inflammation to pathogenic mechanisms in diabetic nephropathy [5]. Macrophage migration and recruitment, especially M1 subpopulations, directly contribute to renal injury in diabetes, perhaps by altering podocyte integrity [6] and directly correlate with the degree of renal fibrosis [7]. Furthermore, several immunomodulatory molecules such as chemokines (CCL2, CCL3L1 and CCL5), adhesion molecules (intercellular adhesion molecule 1, vascular cell adhesion protein 1, E-selectin and α-actinin 4), and cytokines (IL-1, IL-6, IL-18 and tumor necrosis factor-α) have all been implicated in the pathogenesis of diabetic nephropathy [5].

Chitotriosidase enzyme is a fully active human chitinase that was initially discovered to be markedly elevated in the plasma of patients with Gaucher’s disease, an inflammatory-based lysosomal storage disorder [8]. Later, its elevation was detected in other lysosomal and non-lysosomal inflammatory disorders [9–12]. Chitotriosidase is both an endo- and exo-chitinase splitting chitin molecules into smaller polysaccharide moieties and also to its basic monosaccharide N-acetylgalactosamine [13]. The absence of its substrate chitin in mammals and the exclusive expression in immune cells, mainly activated macrophages, elucidated its involvement in the activation...
of the innate and acquired immune systems, especially against chitin coated pathogens [13,14]. Many immune enhancing molecules such as tumor necrosis factor-α, interferon-γ and lipopolysaccharide promote chitotriosidase expression in activated macrophages [15], while chitotriosidase was shown to stimulate IL8, CCL2, CCL5 and etoxacin and to increase the migratory capacity of eosinophils, T lymphocytes and macrophages [16]. Chitotriosidase also stimulates transforming growth factor-β1 receptor expression and signaling suggesting a role in enhancing the response to organ injury and repair. For example, bleomycin-induced pulmonary fibrosis was significantly reduced in chitotriosidase null mice and significantly enhanced in chitotriosidase over-expressing transgenic mice [17].

The 24-bp duplication mutation (rs3831317) at exon ten of the chitotriosidase gene (CHIT1), resulting in aberrant splicing and deletion of 87 nucleotides, is the main cause of complete enzyme deficiency in about 6% of Caucasians when homozygously mutated. It also reduces the chitotriosidase enzyme expression in heterozygous individuals [18]. In this cross-sectional study we aimed to investigate the potential role of chitotriosidase enzyme and its functional mutation (24-bp duplication) in the development and progression of nephropathy in a cohort of Egyptian type 2 diabetic patients.

2. Patients and methods

2.1. Patients

For the current study, 262 Egyptian type 2 diabetic patients (48.6 ± 5 y, 42.3% males) for a minimum duration of ten years, and 90 non-diabetic controls (43.5 ± 4.7 y, 38.9% males) were recruited from patients presenting to the internal medicine and nephrology outpatient clinics at Theodor Billharz research institute (TBRI) and blood donors visiting the blood bank at TBRI respectively. Enrolment of patients extended over the period from October 2013 to December 2014. Diabetic patients were further divided into patients without diabetic nephropathy evidenced by urinary albumin excretion <3.4 mg/mmol creatinine (n = 84, 47.4 ± 5.2 y) and patients with diabetic nephropathy (n = 178, 49.3 ± 4.7 y), presenting with either microalbuminuria (3.4–34 mg/mmol creatinine, n = 88, 47.8 ± 4.1 y) or macroalbuminuria (>34 mg/mmol creatinine, n = 90, 51 ± 4.6 y). Body mass index (BMI) in control subjects was matching type 2 diabetic patients.

Demographic and clinical data were recorded including age, gender, BMI, duration of diabetes and history of previous episodes of myocardial infarction or stroke. Associated hypertension was determined based on standard clinical investigations including fasting plasma glucose, urea, creatinine, total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol, and eGFR. Estimated GFR (eGFR) were assayed by Synchron CX5 (Beckman Coulter, Brea, California, USA), which is a fully automated analyzer that utilizes ultrasensitive enzymatic methods for the determination of creatinine (hexokinase method) and urea (urease/kinase method) to calculate eGFR according to the Modification of Diet in Renal Disease (MDRD) study equation. [19]

2.2. Chitotriosidase enzyme activity

Plasma chitotriosidase activity was measured as described previously [20]. Ten microliters of plasma were mixed with 100 μL of 0.022 mmol/l 4-methylumbelliferyl-D-β-N,N′-triacetylichitotrioside (Sigma) in citrate/phosphate buffer, 0.1/0.2 mol/L, pH 5.2 and incubated at 37 °C for 15 min. The reaction was stopped with 2 mL of 0.5 mol/L carbonate/bicarbonate buffer, pH 10.7. Fluorescence was measured by FP 6200 (Jasco analytical instruments, Tokyo, Japan) at excitation wavelength 435 nm and emission wavelength 448 nm. Enzyme activities were calculated based on a calibration curve of 4-methylumbelliferone and expressed as nmol of enzyme product formed/mL plasma/h.

2.3. Chitotriosidase genotype

DNA was extracted from EDTA blood using the GeneJET DNA purification kit (Thermo Scientific, MA, USA) according to the manufacturer’s protocol. The detection of the 24-bp duplication mutation within exon ten of CHIT1 gene was performed as previously described [18] using the primers 5'-AGCTATCTGAAGCAGAAG-3' and 5'-GGAGAAGCCGGC AAACGTC-3' as forward and reverse primers, respectively. PCR protocol constituted of initial denaturation at 95 °C for 3 min followed by 35 cycles of: denaturation at 95 °C for 30 s, annealing at 56 °C for 60 s, extension at 72 °C for 30 s and final extension at 72 °C for 7 min. Detection of PCR amplification products was performed using either 4% agarose or 15% polyacrylamide gel electrophoresis, ethidium bromide as a staining dye and ultraviolet transillumination: revealing the formation of a 75-bp band in the wild-type, a 99-bp band in the homozygously mutated and both in the heterozygous. Genotype assessment was repeated in a random sample consisting of 25% of all subjects.

2.4. Statistical analysis

Comparisons of quantitative data were analyzed using Mann–Whitney U-test or Kruskal–Wallis test for differences between medians. Pearson chi-square (χ2) test was used to test categorical variables among groups and also to test the Hardy–Weinberg equilibrium for the mutation genotype and allele frequencies among controls and patients. Association of chitotriosidase enzyme activities with the continuous variables urinary albumin/creatinine ratio and estimated GFR was performed through multiple linear regression after adjusting for age, gender, BMI, duration of diabetes, hypertension status, total cholesterol, triglycerides and HbA1c levels. The 24-bp duplication mutation was also associated with the dichotomous variable diabetic nephropathy through multiple logistic regression adjusted for the same confounders. Associations were expressed as regression coefficients with standard errors for linear regressions and as odds ratios with 95% confidence intervals (CI) for logistic regressions. A 2-sided P value < 0.05 was considered significant. Sample size calculation was based on preliminary data obtained from a pilot study performed in 50 type 2 Egyptian diabetic patients, whose data are not included in the current study. Statistical analysis was performed by the WINPEPI statistical software package, version 11.43 [21].

3. Results

The study sample consisted of 262 Egyptian type 2 diabetic patients and 90 non-diabetic controls. Demographic, clinical and biochemical data of study participants are summarized in Table 1. Chitotriosidase enzyme activities (median; 25th–75th percentiles) were significantly elevated in diabetic patients (33; 30–96 nmol/mL plasma/h) as compared to non-diabetic controls (37; 31–46 nmol/mL plasma/h), P < 0.001. Furthermore, there was a significant increase in chitotriosidase
activity among diabetic patients when categorized according to nephropathy status: normoalbuminuria (30; 20–49 mmol/ml plasma/h), microalbuminuria (53; 33–90 mmol/ml plasma/h) and macroalbuminuria (87; 56–165 mmol/ml plasma/h), \( P < 0.001 \) (Fig. 1).

Chitotriosidase levels also correlated significantly with the estimates of progression of kidney disease in diabetic patients performed in our study. The strongest correlation was with urinary albumin creatinine ratio \( (r = 0.783) \), followed by eGFR \( (r = 0.423) \), creatinine \( (r = 0.392) \) and urea \( (r = 0.305) \), \( P < 0.001 \) for each of them. Chitotriosidase activities also correlated with the duration of diabetes \( (r = 0.179, P = 0.003) \). Furthermore, chitotriosidase independently predicted both albumin creatinine ratio and eGFR in the fitted multiple linear regression models after adjustment for the confounders: Age, gender, BMI, duration of diabetes, hypertension status, total cholesterol, triglycerides and HbA1C levels, \( P < 0.001 \) for both. Table 2 summarizes the results of the linear regression models performed. There was no direct correlation between chitotriosidase levels and other quantitative clinical or laboratory measures such as: age, BMI, fasting plasma glucose, HbA1C, ALT, total bilirubin, total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol.

The genotypic and allelic frequencies of the investigated 24-bp duplication mutation in control subjects and in diabetic patients are summarized in Table 3. They were all in accordance with the Hardy–Weinberg equilibrium. The wild genotype was detected more commonly with higher renal damage in diabetic patients, while the mutated was more associated with a lower renal damage. Using the Pearson \( \chi^2 \) test a significant difference existed between the three diabetic groups for both genotypic and allelic frequencies (\( P = 0.007 \) and \( P = 0.009 \), respectively).

In the multiple logistic regression model (Table 4), the 24-bp duplication mutation genotype (WT/Dup and Dup/Dup) was an independent negative predictor of the onset of nephropathy in type 2 diabetic patients after adjustment for the same confounders mentioned previously (OR: 0.517, 95% CI: 0.289–0.924, \( P = 0.026 \)). Diabetic nephropathy also associated negatively with female gender (\( P = 0.045 \)), while there was a positive association with multiple independent factors such as: duration of diabetes (\( P = 0.009 \)), total cholesterol (\( P = 0.012 \)), triglycerides (\( P = 0.017 \)) and HbA1C levels (\( P = 0.035 \)). When diabetic retinopathy, neuropathy or macrovascular complications were set as the dependent variable in the logistic regression model, no significant association was detected between the tested genotype and any of these complications.

4. Discussion

In the current study we evaluated the association of chitotriosidase enzyme and its loss of function mutation (24-bp duplication) with the phenotype features of type 2 diabetes. The chitotriosidase levels were found to be significantly higher in the diabetic group compared to the non-diabetic controls. This finding is in line with previous studies, which reported increased chitotriosidase activity in diabetic patients compared to healthy controls [5,6].

In the current study, we found that diabetic patients had higher chitotriosidase activity compared to the non-diabetic controls. This finding is consistent with previous studies [5,6]. The increased chitotriosidase activity in diabetic patients might be due to the up-regulation of the enzyme in response to the chronic inflammation associated with diabetes [7]. The increased chitotriosidase levels observed in diabetic patients might also be due to the increased production of the enzyme in response to the chronic inflammation associated with diabetes [7].

The current study also found that diabetic patients had higher chitotriosidase activity compared to the non-diabetic controls. This finding is consistent with previous studies [5,6]. The increased chitotriosidase activity in diabetic patients might be due to the up-regulation of the enzyme in response to the chronic inflammation associated with diabetes [7]. The increased chitotriosidase levels observed in diabetic patients might also be due to the increased production of the enzyme in response to the chronic inflammation associated with diabetes [7].

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Table 2

Multiple linear regression analysis of the relation of chitotriosidase enzyme activity to urinary albumin/creatinine ratio and eGFR.

<table>
<thead>
<tr>
<th>Variable</th>
<th>β ± SE of β</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Dependent: urinary albumin/creatinine ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Independent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.656 ± 3.689</td>
<td>0.859</td>
</tr>
<tr>
<td>Gender (0 = male, 1 = female)</td>
<td>4.744 ± 21.648</td>
<td>0.827</td>
</tr>
<tr>
<td>BMI</td>
<td>3.830 ± 4.450</td>
<td>0.390</td>
</tr>
<tr>
<td>Diabetes duration</td>
<td>10.721 ± 4.342</td>
<td>0.014</td>
</tr>
<tr>
<td>Hypertension (0 = absent; 1 = present)</td>
<td>−21.65 ± 27.078</td>
<td>0.425</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.118 ± 0.189</td>
<td>0.533</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.076 ± 0.175</td>
<td>0.664</td>
</tr>
<tr>
<td>Hba1c</td>
<td>3.192 ± 3.959</td>
<td>0.806</td>
</tr>
<tr>
<td>Chitotriosidase activity</td>
<td>3.067 ± 0.156</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B. Dependent: eGFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Independent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.309 ± 0.768</td>
<td>0.688</td>
</tr>
<tr>
<td>Gender (0 = male, 1 = female)</td>
<td>−3.770 ± 4.494</td>
<td>0.402</td>
</tr>
<tr>
<td>BMI</td>
<td>1.232 ± 0.924</td>
<td>0.148</td>
</tr>
<tr>
<td>Diabetes duration</td>
<td>−3.152 ± 0.901</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension (0 = absent; 1 = present)</td>
<td>−5.137 ± 5.022</td>
<td>0.362</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>−0.094 ± 0.039</td>
<td>0.017</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>−0.078 ± 0.036</td>
<td>0.032</td>
</tr>
<tr>
<td>Hba1c</td>
<td>−0.693 ± 0.622</td>
<td>0.400</td>
</tr>
<tr>
<td>Chitotriosidase activity</td>
<td>−0.221 ± 0.032</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

β, regression coefficient; SE, standard error.

risk of development and progression of nephropathy in a large cohort of type 2 diabetic patients.

Being an active chitinase, the wild type and fully active human chitotriosidase enzyme is essential for the resistance against many chitin coated pathogens such as Plasmodium falciparum [22], Wuchereria bancrofti [23], Cryptococcus neoformans and Candida albicans [24]. This is evidenced by the almost complete absence of the 24-bp duplication mutation of CHIT1 gene in sub-Saharan Africa (0–2% of individuals) confirming the evolutionary advantage of the wild type enzyme in areas with high endemic and parasitic disease loads [25]. On the other hand, the high prevalence of the mutated genotype in European countries (30–40% of individuals) with much lower rates of endemic and parasitic diseases [18,25] must also point to a different evolutionary advantage for the mutated. For example, it is well known that individuals of African ancestry are more prone to develop nephropathy and ESRD complicating diabetes or other different pathological conditions. This is regardless of social status or the level of health care coverage supporting the role of genetic predisposition in the development and progression of kidney disease in this ethnic group [26]. Could this be related, at least partially, to the abundance of the wild type CHIT1 gene in African populations compared to Caucasians? This is yet to be determined.

Furthermore, chitotriosidase genotype has been linked to the pathogenesis of many other inflammatory based conditions. Di Rosa et al. elucidated the protective role of the 24-bp duplication mutation of the CHIT1 gene against the development of non-alcoholic fatty liver disease in Italian population [27]. In another study, Kim et al. associated the gain of function mutation (A442G) in the CHIT1 gene with a higher risk of atopy in Korean children [28]. Malguarnera et al. also associated the 24-bp duplication heterozygous genotype positively with human longevity in three Mediterranean populations (Italian, Greek and Tunisian) [29]. Denoting in accordance with the current study results that limiting chitotriosidase expression might be beneficial in more than one aspect.

Few studies have previously explored the correlation of chitotriosidase enzyme with diabetes. Sommez et al. correlated chitotriosidase activity with the asymmetric dimethyl arginine levels in plasma of newly diagnosed and uncomplicated patients with type 2 diabetes and concluded that chitotriosidase enzyme could be a predictor of endothelial dysfunction in those patients [30]. Kabaroglu et al. further confirmed that chitotriosidase levels were significantly higher in obese adolescents with glucose intolerance compared to a matched group with normal glucose tolerance [31]. Elmonem et al. detected significant elevations of chitotriosidase levels in diabetic patients with renal impairment versus healthy individuals when both groups were taken as comparators for a cohort of nephropathic cystinosis patients [10]. Recently, Żurawska-Plaskiej et al. also showed that chitotriosidase activity together with the concentration of another member of the 18 glycosyl hydrolase protein family (YKL-40) correlated with the degree of renal insufficiency in type 2 diabetic subjects [32]. In the current study, we confirmed our previous results [10] and the results of Żurawska-Plaskiej et al. [32] in a much bigger cohort (almost triple) and we further elucidated the role of the functional 24-bp duplication mutation in decreasing the risk of developing nephropathy in type 2 diabetic patients suggesting an important role played by chitotriosidase in the pathogenesis of diabetic nephropathy.

The fact that chitotriosidase levels were not significantly different between non-diabetic controls and normoalbuminuric diabetic patients in our study (Fig. 1) denotes that the enzyme levels are mostly associating between non-diabetic controls and normoalbuminuric diabetic patients suggesting an important role played by chitotriosidase genotype and activity with the onset and progression of diabetic nephropathy.

Table 3

Frequencies of wild type and mutant genotypes and alleles in control subjects and different diabetic groups.

<table>
<thead>
<tr>
<th>Genotype frequency</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wt/ Wt/dup Dup/dup</td>
</tr>
<tr>
<td>Controls (n = 90)</td>
<td>53 (58.9%)</td>
</tr>
<tr>
<td>(n = 84)</td>
<td>41 (48.8%)</td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>54 (61.4%)</td>
</tr>
<tr>
<td>(n = 88)</td>
<td>37 (43.1%)</td>
</tr>
<tr>
<td>Macroalbuminuria</td>
<td>65 (72.2%)</td>
</tr>
<tr>
<td>(n = 90)</td>
<td></td>
</tr>
</tbody>
</table>

Wt: wild type; Dup: 24-bp duplication mutation.

CHIT1 gene against the development of non-alcoholic fatty liver disease in Italian population [27]. In another study, Kim et al. associated the gain of function mutation (A442G) in the CHIT1 gene with a higher risk of atopy in Korean children [28]. Malguarnera et al. also associated the 24-bp duplication heterozygous genotype positively with human longevity in three Mediterranean populations (Italian, Greek and Tunisian) [29]. Denoting in accordance with the current study results that limiting chitotriosidase expression might be beneficial in more than one aspect.

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The fact that chitotriosidase levels were not significantly different between non-diabetic controls and normoalbuminuric diabetic patients in our study (Fig. 1) denotes that the enzyme levels are mostly associated with the onset of glomerular insult rather than the earlier tubular phase of the disease. This might be explained by the timing of influx of inflammatory cells to the renal parenchyma which is usually shortly preceding the glomerular insult [6,7].

An important limitation of our study is lacking the smoking history input into the regression models performed. Several previous studies associated smoking and oxidative stress propagated by smoking to the development of nephropathy in diabetic patients [33,34]. The prevalence of smoking in the Egyptian community is by far higher in males than females, as it is considered sort of socially and ethically unacceptable behavior for females. We believe that it is highly unlikely that smoking will affect the strong association between chitotriosidase genotype and activity with the onset and progression of diabetic nephropathy in type 2 diabetes, Clin Biochem (2015), http://dx.doi.org/10.1016/j.clinbiochem.2015.11.009
nephropathy detected in our study, but it might confound the female gender protective role detected in the logistic regression model (Table 4).

In conclusion, we confirmed for the first time the strong association between chitinotriosidase enzyme activity and the markers of progressive kidney disease in a large cohort of type 2 diabetic patients. We also demonstrated that diabetic patients genetically lacking the full active enzyme (with at least one CHIT1 functionally mutated allele) are at lower risk of developing nephropathy compared to patients having the wild genotype. Both findings strongly implicate chitinotriosidase involvement in the pathogenic pathway leading to the development and progression of nephropathy in type 2 diabetic patients.

Conflict of interest

The authors declare no conflict of interest regarding this manuscript.

Acknowledgments

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