Heat-shock protein gene polymorphisms and the risk of nephropathy in type 2 diabetes patients

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ABSTRACT

Heat-shock proteins (HSPs) are molecular chaperones synthesized under stress conditions. HSPs are involved in renal cell survival and matrix remodeling in acute and chronic renal diseases. We investigated whether the HSP70 gene polymorphisms affect susceptibility to nephropathy in type 2 diabetes patients.The study group consisted of 452 patients with nephropathy. Two control subgroups involved: 340 healthy individuals and 132 patients with type 2 diabetes lasting ≥ 10 years, free of nephropathy. Subjects were genotyped for the HSP70-1 +190 G/C and -110 A/C, HSP70-2 +1267 A/G and HSP70-hom +2437 T/C polymorphisms by polymerase chain reaction (PCR) followed by digestion with restriction endonucleases. There were no statistically significant differences in genotype distribution between diabetic nephropathy (DN) patients and controls for the HSP70-hom polymorphism. Significant differences were observed for HSP70-1 and HSP70-2 polymorphisms. The CC homozygotes of the -110 and +190 HSP70-1 polymorphisms were more frequent in DN patients than in healthy controls (22 vs. 6 % and 15 vs. 6.5 %, respectively, $p<0.01$). The OR for the risk allele was 2.17 (95 % CI 1.73-2.72) for the -110 A/C and 1.74 (95 % CI 1.40-2.15) for +190 G/C polymorphisms. A strong association with DN was found for the +1267 HSP70-2 polymorphism. The GG genotype and the G allele were associated with DN, with OR for the G allele 4.77 (95 % CI 3.81-5.96). All GG homozygotes in patient group had higher LDL cholesterol level than AA homozygotes ($p<0.01$), suggesting that the observed effect might be associated with cardiovascular risk factor. These patients progressed faster to end-stage renal failure than those with other genotypes. Our results indicate that the HSP70-1 and HSP70-2 polymorphisms are associated with renal complications in type 2 diabetes and may be useful in identifying patients with increased risk of diabetic nephropathy.

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INTRODUCTION

Diabetic microvascular complications are the major cause of morbidity and premature mortality in type 2 diabetes [1,2]. Diabetic nephropathy has become the leading cause of end-stage renal failure worldwide [3,4]. There is a large body of evidence implicating genetic factors in the susceptibility to diabetic nephropathy and retinopathy [5-7]. Numerous polymorphisms in the candidate genes have been investigated in relation to diabetic complications [6].

Heat-shock proteins (HSPs) are molecular chaperones synthesized under stress conditions. They are induced by denatured proteins during heat shock, ischemia and other cellular stresses. HSPs are important in physiological and pathological processes and are highly active within the immune system [8]. They help in restoring protein homeostasis and assist in cellular recovery from stress by repairing damaged proteins through refolding or by degrading them [9]. Oxidant stress plays an important role in renal diseases [10]. Recent studies have documented the crucial role of HSPs in renal cell survival and matrix remodeling in several acute and chronic renal diseases [11]. They have also been reported to be involved in diabetes through their effect on insulin sensitivity [8].

Heat-shock protein 70 kD (HSP70) family is the most abundant in eukaryotic cells and is essential for cell survival under stress conditions [12]. In humans, three genes encoding members of the HSP70 class are mapped within major histocompatibility complex class III region (6p21.3) : HSP70-1, HSP70-2 and HSP70-hom. The HSP70-1 and HSP70-2 encode an identical heat-inducible protein HSP70 but differ in their regulatory domains whereas HSP70-hom encodes a non-heat inducible form [13]. These genes are polymorphic, with some variants potentially accounting for a change in function and susceptibility to stress tolerance [14,15]. HSP70 gene polymorphisms were found to be risk factors in several human disorders [15-18]. They might play an important role in susceptibility to and/or progression of diabetic nephropathy.

To address this possibility, we investigated the potential involvement of gene variants of HSP70-1, HSP70-2 and HSP70-hom in diabetic nephropathy in type 2 diabetes patients.

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MATERIALS AND METHODS

Study participants

The study population consisted of 452 unrelated type 2 diabetes patients with nephropathy, 132 patients with type 2 diabetes duration of 10 or more years, but free of nephropathy (30 % of those had retinopathy) and 340 healthy individuals. All subjects were Caucasians of Polish origin. Diabetic nephropathy status was determined on the basis of questionnaires, medical files and laboratory data. The albumin excretion rate (AER) was determined in three 24-h urine collections performed at least 1 month apart. Diabetic nephropathy was diagnosed clinically when the patient had persistent albuminuria ≥ 300 mg/24 hrs in at least two consecutive determinations, in the absence of hematuria or infection. Repeated measurements of the albumin / creatinine ratio (ACR) were performed. Overt proteinuria was defined as an ACR >28.2 mg/g in men and > 40.2 mg/g in women. In addition, the patients had no clinical, laboratory or radiological findings indicating any secondary form of nephropathy. All patients with diabetic nephropathy were undergoing maintenance dialysis. The mean duration of diabetes was 13.6 years (range 9-26). The patient subgroup without DN consisted of subjects who had diabetes duration ≥ 10 years and ACR < 1.9 mg/g in men or < 2.8 mg/g in women in at least two determinations. All patients with microalbuminuria were excluded from the study. In the patient group 392 individuals (67 %) were hypertensive (systolic blood pressure > 140 mm Hg and diastolic blood pressure > 90 mm Hg) and receiving antihypertensive treatment. Seventy two percent of diabetic nephropathy patients were also diagnosed with retinopathy. A positive family history of diabetes in first-degree relatives was reported by 140 patients (24 %).

Control subjects (n = 340) were healthy volunteers (mostly blood donors and hospital staff) with no history of diabetes and renal or cardiovascular disease. The urine analysis was performed in all subjects. Over 40 % of control subjects had the oral glucose tolerance test Written informed consent was obtained from all subjects enrolled in the study in accordance with principles of the Declaration of Helsinki. The protocol of the study was approved by the institutional ethics committee.

Determination of HSP70 genotypes

Genomic DNA was extracted from peripheral blood leukocytes using the method described by Madisen et al. with minor modifications [19]. Genotypes for HSP70-1, HSP70-2 and HSP70-hom polymorphisms were determined by PCR-RFLP method (Table 1). Amplified PCR fragments were digested overnight with appropriate restriction endonucleases (Fermentas GmbH, St Leon-Rot, Germany) and separated on 2 % agarose gels.

Statistical analysis

Statistical calculations were performed using SPSS 9.0 for Windows (SPSS, Inc., Chicago, IL, USA). Normally distributed data are presented as means ± SD. The Hardy-Weinberg equilibrium was tested with the X^2 test. Genotype distribution and allele frequencies were compared between groups using a X^2 test of independence with 2x2 contingency and *z* statistics. Student's t-test and Man-Whitney test were used for statistical significance. Where appropriate, the odds ratios (OR) with 95 % confidence intervals (CI) were calculated. A two-tailed type I error rate of 5 % was considered statistically significant. Multivariate logistic regression with two-way analysis was performed for analysis of independent risk factor for diabetic nephropathy. Power calculations were done using on-line available power calculator (http://calculators.stat.ucla.edu).

RESULTS

The demographic and clinical profiles of studied patients and controls are presented in Table 2. No statistically significant differences in the clinical characteristics were observed between patients with diabetic nephropathy and those without nephropathy. The gender distribution was similar in both diabetic and healthy groups. All patients and control subjects were genotyped for the HSP70-1 -110 A/C and +190 G/C; HSP70-2 +1267 A/G and HSP70-hom +2437 T/C polymorphisms.

HSP70-1 gene polymorphisms

The frequency of genotypes for HSP70-1 gene polymorphisms did not deviate significantly from the Hardy-Weinberg equilibrium. The distribution of the genotypes

and alleles of the -110 A/C and +190 G/C polymorphisms in patients and controls is shown in Table 3. For both polymorphic sites significant differences in genotype distribution between patients with nephropathy and controls were observed. The odds ratio for the minor allele (C in both cases) was : for -110 A/C 2.17 (95 % CI 1.73-2.72, p<0.01) and for +190 G/C 1.74 (95 % CI 1.40-2.15, p<0.01). Odds ratios calculated for homozygous CC genotypes of both polymorphisms, with homozygotes for major allele as a reference, suggest that both CC genotypes might be genetic risk factors for diabetic nephropathy. The observed effect is not confined to homozygous genotype (Table 4). No statistically significant differences in genotype distribution and allele frequencies were observed between diabetic patients without nephropathy and controls. Since a proportion of patients classified as diabetic nephropathy might have a non-diabetic renal disease, we also calculated the genotype/allele frequencies for patients with both diabetic nephropathy and retinopathy (data not shown). In this subgroup we still observed association between nephropathy and genotype, with the OR = 1.99 (1.31-2.43).

The risk of diabetic nephropathy related to the C allele of -110 A/C and the C allele of +190 G/C polymorphism remained unchanged after multivariate logistic regression analysis with age, gender, diabetes duration, HbA1c and hypertension as covariates.

HSP70-2 +1267 A/G polymorphism

The distribution of genotypes and alleles of this polymorphism is shown in Table 5. The GG genotype and G allele were significantly associated with diabetic nephropathy, with the OR for the G allele 4.77 (95 % CI 3.81-5.96) There was no statistically significant difference between two control groups - healthy individuals and type 2 diabetes patients without nephropathy.

All GG homozygotes in the patient group had higher total cholesterol and LDL cholesterol levels than AA homozygotes (236 ± 41 vs. 189 ± 33 mg/dL and 141 ± 25 vs. 117 ± 29 mg/dL, respectively, p<0.01). This suggests that observed effect of the +1267 polymorphism might be associated with cardiovascular risk factor.

We have preliminary observations suggesting some effect of the GG homozygosity on the progression of diabetic nephropathy to ESRD. The mean time from the diagnosis of nephropathy to ESRD was 4.4 years for the GG homozygotes compared to 8.7 years for the AA homozygotes (p<0.001). These effect was not observed for

the HSP70-1 gene polymorphisms. This observation will be a subject of our further studies.

HSP70-hom +2437 T/C polymorphism

There was no statistically significant difference in genotype distribution between diabetic nephropathy patients, diabetic patients without nephropathy and healthy controls (data not shown).

DISCUSSION

Heat-shock proteins synthesized under stress conditions are involved in several human diseases. There are reports suggesting an important role of HSP70 proteins in type 1 diabetes with nephropathy [20], type 2 diabetes [21] and vascular events in type 2 diabetes patients [22].

To our knowledge ours is the first study showing a significant association between the HSP70 gene polymorphisms and diabetic nephropathy in type 2 diabetes patients. We hypothesized that genetic variations in the HSP70 genes might affect the risk of microvascular complications in type 2 diabetes. Two hundred fifty two patients with type 2 diabetes were genotyped with HSP70-1, HSP70-2 and HSP-hom polymorphisms. The results show that HSP70-1 gene polymorphisms at positions -110 and +190 and HSP70-2 gene variant at position +1267, a silent change in the coding region, are associated with nephropathy in type 2 diabetes patients. It should be mentioned that all DN patients in our study had end-stage renal disease due to diabetic nephropathy so we did not study a relationship between genotype and a stage of nephropathy.

Variations -110 A/C and +190 G/C are in the 5' flanking and non-coding regions of the HSP70-1 gene and were earlier reported to be associated with Parkinson's disease [15] , celiac disease [23] and autoimmune thyroid disease[24]. The -110 A/C polymorphism is located 3 bp upstream of the heat-shock element essential for the heat inducibility of the HSP70-1 gene. It also lies within the five consecutive regulatory elements involved in the binding of regulatory protein [25]. It is thus possible that -110 A/C alters regulation of the HSP70-1 gene.

Both variations in the HSP70-1 gene, -110 A/C and + 190 G/C, seem to be genetic risk factors for diabetic nephropathy, with similar odds ratio for the minor allele (2.17

and 1.74, respectively) . In fact, a strong linkage disequilibrium was reported between -110 A/C and +190 G/C and between HSP70-1 -110 A/C and HSP70-2 +1267 A/G [15, 26].

The two polymorphisms of the HSP70 genes, HSP70-1 -110 A/C and HSP70-2 +1276, found in our study to be associated with diabetic nephropathy, may be related to the disease through similar mechanisms. It was reported earlier that the HSP70-1 -110C and HSP70-2 +1267G alleles are strongly non-randomly associated with each other [26]. These two HSP70 genes share a similar heat-shock element, what suggests their similar affinities for heat-shock factor. The HSP70-1 and HSP70-2 genes also share the same transcriptional start site [13] . The sequence differences in 5' flanking regions suggest that these genes may be differentially regulated in response to stress factors other than heat shock. Pociot et al. reported the association of variable HSP70-2 mRNA expression and the +1267 polymorphism [27]. In subjects with homozygous GG genotype a decrease of mRNA expression might occur and the cell response to stress would be impaired, leading to the intracellular accumulation of denaturalized proteins or peptide transporting defects. Sequence variations that result in altered expression or protein activity could determine susceptibility to disease.

It is possible that the HSP70-2 polymorphism is not involved causally in contributing to diabetic nephropathy, but is in a linkage disequilibrium with some neighboring gene on chromosome 6p.

In our diabetic nephropathy patient group all GG homozygotes of the HSP70-2 +1267 polymorphism had higher total cholesterol and LDL cholesterol levels than AA homozygotes. Thus the observed effect of the polymorphism might be associated with increased risk of cardiovascular disease. This is in agreement with the study of Giacconi et al. The authors demonstrated that total cholesterol and LDL cholesterol concentrations were significantly higher in B+ (presence of a G allele) than in B- NIDDM atherosclerotic patients [22].

The HSP70-hom +2437 T/C polymorphism (Met→Thr amino acid substitution at position 493) was reported to be associated with spondyloarthropathies [16] and sarcoidosis [17]. It was also found to affect insulin-dependent diabetes mellitus [27]. It is thought that this substitution may be associated with variation in the peptide-binding specificity of different HSP70-hom haplotypes. In our study this polymorphism did not show any effect on susceptibility to diabetic nephropathy. In another study it

was investigated along with HSP70-1 and HSP70-2 gene polymorphisms and was not involved in the susceptibility to Parkinson's disease [15].

The associations between the HSP70 gene polymorphisms and diabetic nephropathy, shown in our study, are not observed for diabetes itself.

The strengths of our study are that all patients and controls are of the same ethnic origin. Furthermore, all subjects were examined in a standardized manner, with well defined diagnostic criteria. All genotyping was performed blind with respect to case-control status. However, the genetic heterogeneity and small effects of some disease associated alleles make association studies difficult in multifactorial diseases with complex phenotypes. The occurrence of diabetes and/or its complications depends on the interaction among the presence of different risk alleles, environmental factors and the lifestyle. The influence of any single polymorphism is rather small and an interactive effect of several factors may lead to an underestimation or an overestimation of a role of given polymorphism in determining the phenotype.

In conclusion, our data suggest that HSP70-1 and HSP70-2 gene polymorphisms are associated with predisposition to diabetic nephropathy in type 2 diabetes patients, at least in Polish population. It is a novel observation which needs to be confirmed in additional studies in a large number of patients and control subjects. Also the functional studies are needed to elucidate the involvement of HSP70 genes in diabetic microvascular complications. If observed associations are confirmed, the HSP70 gene polymorphisms may be useful in identifying diabetic patients with an increased risk of nephropathy.

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***Conflict of interest statement*.** None declared.

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Table 1 **HSP70 primer sequences, PCR conditions and product detection**

Gene	Primers	PCR conditions MgCl2 / annealing temp.	SNP	Enzyme Fragm. size (bp)
HSP70-1	F : CGCCATGGAGACCAACACCC R : GCGGTTCCCTGCTCTCTGTC	1.0 mM 60°C	- 110 A/C + 190 C/C	Sac I 215, 201, 72 Mbi I / 461,27 / 488
HSP70-2	F : CATCGACTTCTACACGTCCA R : CAAAGTCCTTGAGTCCCAAC	1.5 mM 60°C	+ 1267 A/G	Pst I / 1118 / 934, 184
HSP70-hom	F : GTCCCTGGGGCTGGAGACGG R : GTGATGATAGGGTTACACATCTGCT	1.0 mM	+ 2437 T/C	Nco I / 354,273 / 627

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Table 2 **Demographic and clinical profile of studied subjects**

For continuous characteristics , values are presented as means ± SD. For discrete characteristics values are numbers and percentages (in parentheses). * median (25-75 % quartiles).

	DM2 patients with DN	DM2 patients no DN	Healthy controls
N	452	132	340
Sex (M / F)	253 / 199	69 / 63	171 / 169
Age at study (years)	54.9 ± 17	55.9 ± 14	55 ± 16
Diabetes duration (years)	14 (10-16)*	12 (8-14)*	NA
Total cholesterol (mmol/l)	5.1 ± 1.22	4.7 ± 1.7	4.6 ± 1.11
HbA_{1c} (%)	8.8 ± 3.1	8.2 ± 4.7	ND
BMI (kg / m^2)	28.2 ± 3.9	27.9 ± 4.3	25.9 ± 3.8
Hypertension (%)	302 (67)	90 (68)	0
Family history of DM (%)	109 (24)	31 (23.5)	15 (4.4)

DM2, type 2 diabetes; DN, diabetic nephropathy; NA, not applicable; ND, not determined

Clinical Science Immediate Publication. Published on 30 May 2008 as manuscript CS20070411

Table 3 Distribution of HSP70-1 polymorphisms in diabetic nephropathy patients and controls

Data are n (%). * p<0.01 (after Bonferroni correction) versus DN.

SNP	DM2 with DN (n = 452)	DM2 no DN (n = 132)	Controls (n = 340)
-110 A / C			
AA	129 (29)	66 (50)	161 (47.5)
AC	222 (49)	56 (42.5)	158 (46.5)
CC	101 (22)	10 (7.5) *	21 (6) *
A allele	0.53	0.71	0.71
C allele	0.47	0.29	0.29
Power 98.4 %			
+190 G / C			
GG	149 (33)	63 (48)	168 (49.5)
GC	235 (52)	58 (44)	150 (44)
CC	68 (15)	11 (8)*	22 (6.5)*
G allele	0.59	0.70	0.71
C allele	0.41	0.30	0.29
Power 96.1 %			

DM2, type 2 diabetes; DN, diabetic nephropathy.

Clinical Science Immediate Publication. Published on 30 May 2008 as manuscript CS20070411

Table 4 Association of HSP70-1 gene polymorphisms with DN : Odds ratios for minor alleles and their homo- and heterozygous genotypes

* vs. healthy controls; † vs. homozygosity for major allele

SNP	Allele * / Genotype†	OR	95% CI	P
-110 A/C	C	2.17	1.73-2.72	< 0.01
	AA	1.00	-	
vs. controls	AC	1.75	1.28-2.38	< 0.01
	CC	6.00	3.55-10.13	< 0.001
	C	2.18	1.62-2.93	< 0.01
	AA	1.00	-	
vs. no DN	AC	2.02	1.33-3.07	< 0.01
	CC	5.16	2.52-10.55	0.003
+190 G/C	C	1.74	1.40-2.15	< 0.01
	GG	1.00	-	
vs. controls	GC	1.76	1.30-2.38	< 0.01
	CC	3.48	2.05-5.91	0.0013
	C	1.60	1.19-2.14	< 0.01
	GG	1.00	-	
vs. no DN	GC	1.71	1.13-2.58	< 0.01
	CC	2.61	1.29- 5.27	0.006

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Table 5 Distribution of HSP70-2 +1267 polymorphism in diabetic nephropathy patients and controls

Data are n (%). * p < 0.01 (after Bonferroni correction) vs. DN patients. Odds ratio (OR) for the G allele is 4.77 (95% CI 3.81-5.96), p<0.001 vs. DN. OR for genotypes with G allele is 24.16 (13.66-42.72) for GG and 5.59 (3.95-7.92) for AG, vs. controls (p<0.001) and 11.46 (5.96-22.0) for GG and 5.36 (3.40-8.45) for AG, vs. no DN patients (p<0.01).

		DM2 with DN (n = 452)	DM2 no DN (n = 132)	Controls (n = 340)
+1267 A/G				
Genotype	AA	72 (16)	74 (56)	204 (60)
	AG	235 (52)	45 (34)	119 (35)
	GG	145 (32)	13 (10)*	17 (5)*
Allele	A	0.42	0.73	0.76
	G	0.58	0.27*	0.24*
Power 99.7 %				

DM2, type 2 diabetes; DN, diabetic nephropathy.