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CLINICAL STUDY

The Influence of G-Protein β_3-Subunit Gene and Endothelial Nitric Oxide Synthase Gene in Exon 7 Polymorphisms on Progression of Autosomal Dominant Polycystic Kidney Disease

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ABSTRACT

Background: A significant phenotypical variability is observed in autosomal dominant polycystic kidney disease (ADPKD). The variability cannot be fully explained by the genetic heterogeneity of the disease. We examined the influence of G-protein β_3-subunit C825T polymorphism and endothelial nitric oxide synthase Glu298Asp polymorphism on the progression of ADPKD towards end stage renal failure (ESRF). *Methods:* 306 ADPKD patients (pts) were analyzed; 261 pts (136 males, 125 females) with ESRF, with subgroup of 73 pts (44 males, 29 females) with ESRF before 45 years (rapid progressors), 46 pts (20 males, 26 females) with ESRF later than in 63 years (slow progressors) and 45 ADPKD pts (17 males, 28 females) in mean age 51 years with serum creatinine under 110 μmol/L (slow progressors) and 100 genetically unrelated healthy Czech subjects. DNA samples from collected blood were genotyped for G-protein β_3-subunit C825T genotype in exon 10 and for endothelial nitric oxide synthase Glu298Asp genotype in exon 7. *Results:* The G-protein β_3-subunit C825T genotype exhibited no significant differences among the groups of slow progressors (6.6% (6/91) TT, 54.9% (50/91) CT, 38.8% (35/91) CC), rapid progressors (9.6% (7/73) TT, 46.6% (34/73) CT, 43.8% (32/73) CC), ADPKD group with ESRF between 40–63 years (9.2% (13/142) TT, 50% (71/142) CT, 40.8% (58/142) CC) and control group (12% TT, 44% CT, 44% CC). When comparing the ages of ESRF of all patients with ESRF, we did not find significant differences in the ages: males TT—51.7±8.8 years, CT—51.9±10.3 years, CC—49.7±10.2 years and females TT—56±9.9 years, CT—53.2±8.5 years, CC—53.9±8.7 years. The endothelial nitric oxide synthase

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Glu298Asp and Asp29Asp genotypes were significantly more frequent in rapid progressors (9.6% (7/73) Asp/Asp, 39.7% (29/73) Asp/Glu, 50.7% (37/73) Glu/Glu) and in ADPKD group with ESRF between 40–63 years (11.3% (16/142) Asp/Asp, 41.5% (59/142) Asp/Glu, 47.2% (67/142) Glu/Glu) in comparison with slow progressors (8.8% (8/91) Asp/Asp, 24.2% (22/91) Asp/Glu, 67.0% (61/91) Glu/Glu) and with control group (8% Asp/Asp, 32% Asp/Glu, 60% Glu/Glu) (Chi-square test, $p<0.05$). Comparing the ages of ESRF of all patients with ESRF, we did not find significant differences in the ages in males with Asp/Asp—54.9±10.4 years, Asp/Glu—50.2±9.4 years, Glu/Glu—51.0±10.4 years. We found out in homozygous Asp/Asp females significantly earlier onset of ESRF (49.2±5.6 years) in comparison with heterozygous females (53.3±7.2 years) and with Glu/Glu homozygous females (54.8±9.7 years) (*t*-test, $p<0.05$). *Conclusion:* We excluded the significance of G-protein β_3-subunit C825T polymorphism on the progression of ADPKD. We established the negative prognostic value of the carriers of Asp variant of eNOS polymorphism. Finding of new modifiers could have in future clinical consequences for ADPKD patients.

Key Words: G-protein β_3-subunit C825T polymorphism; Endothelial nitric oxide synthase Glu298Asp polymorphism; Autosomal dominant polycystic kidney disease; Progression.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most frequent inherited renal disorder (prevalence 1:400–1:1000). It is characterized by the development of multiple cysts in both kidneys. The cysts result over decades in chronic renal failure in about 50% of patients by the age 60 years.[1] ADPKD is responsible for 10% of the patients requiring renal replacement therapy.

Its clinical course is highly variable. 85% cases are caused by the mutation of PKD1 gene on chromosome 16, in about 14% cases the PKD2 gene on chromosome 4 is mutated. The mean age of end stage renal failure (ESRF) is 54 years in PKD1 patients and 73 years in PKD2 patients.[2] Mutations in at least one other unidentified gene are responsible in the remainder.[3]

Different mutations in PKD genes may have different effects on clinical course of the disease. Very early onset of the disease has been described in families with mutations in exons 15 and 41 of the PKD1 gene.[4,5] As for PKD2 patients, mutations at the 3′ end of the PKD2 gene result in a milder clinical course.[6] Nevertheless, the clinical variability (interfamilial and intrafamilial) cannot be fully explained by different mutations in these two genes. Factors such as hypertension, hematuria, and urinary tract infection are known to have a negative influence on the progression. The influence of genetic and environmental modifiers on the progression has not been clarified yet. Another factor affecting disease progression is the occurrence of somatic mutations in alleles inherited from healthy parent. Modifying genes might directly affect the function of polycystics by influencing the rate of somatic mutations or the rate of protein interactions. Moreover, they might affect the cystogenesis itself or affect the clinical factors associated with the disease progression.

The environmental factors could also affect the progression of ADPKD. Caffeine was stated as a risk factor for cystogenesis on epithelial cells from human ADPKD cysts.[7] Caffeine increases the accumulation of adenosine 3′, 5′ cyclic monophosphate, which activates the cellular proliferation and increases transepithelial fluid secretion in cysts by increasing the phosphorylation of extracellular signal-regulated kinase. Smoking was established as a risk factor of ESRF.[8] 28 patients with ADPKD were investigated as a model for non-inflammatory renal disease.

It has been hypothesized that some gene variants, which may play a role in the pathogenesis of hypertension and atherosclerosis, could influence the progression of ADPKD. We examined the influence of the polymorphism (C825T) encoding β_3-subunit of heterotrimeric G proteins (GBN3) on chromosome 12 on the progression of ADPKD towards end stage renal failure (ESRF). 825 T allele of GBN3 is associated with the occurrence of the splice variant GBN3s with 41 amino acids deleted. T allele causes the enhanced signal transduction via pertussin toxin-sensitive G protein.[9] T allele was found to be associated with hypertension in whites and blacks.[10,11] Hypertension is an important negative prognostic factor of ADPKD. Hypertension precedes the deterioration of renal

function in about 70% of ADPKD patients. Furthermore, T allele is associated with obesity,[12] which may also affect blood pressure variation. On the other hand, the low renin activity described in the T-allele carrier's individuals may have positive effect on the progression of polycystic kidney disease. Activation of RAS (renin-angiotensin-aldosterone system), presents already in early stages of ADPKD, could promote the cyst growth through the ability of angiotensin II to potentiate the growth of tubular epithelial cells.[13] A few studies have demonstrated increased plasma renin activity in hypertensive ADPKD patients compared to essential hypertensives and in normotensive ADPKD patients compared to normal controls.[14,15] In addition, plasma aldosterone concentration was significantly higher in normotensive ADPKD patients than in normal subjects in the upright position.[15] Moreover, intrarenal angiotensin II levels are much higher than plasma level. Inappropriate functioning of the RAS is consistent with increase in renal vascular resistance.[15–17] The growth and expansion of cysts leads to renal ischemia of renal tissue probably by stretching renal vessels. The focal renal ischemia activates juxtaglomerular apparatus leading to renin synthesis.[18] In ADPKD kidneys, there was also found the expression of renin by arterioles adjacent to cysts and the epithelium of cysts is also able to synthesize renin.[18–20]

As the second polymorphism, we examined the influence of eNOS Glu298Asp polymorphism in exon 7 on chromosome 7 on the progression of ADPKD. NO synthesis by the vascular endothelium is important for the regulation of vasodilator tone and the control of blood pressure in humans.[21] Lower plasma NO level in Asp 298 variant is associated with hypertension in males and females,[22,23] myocardial infarction.[24] ADPKD is associated with an alteration of endothelium-dependent vasodilation, caused by a decreased production of NO by eNOS.[25]

METHODS

Patients

A total number of 306 Czech patients (pts) with ADPKD (153 males, 153 females) entered into this study. After the approval of Czech Ethical Committee in Czech Republic, nephrologists of 22 dialysis centres and 1 transplantation centre in our country were asked for their co-operation. Informed consent was obtained from all patients included. The diagnosis of ADPKD was established according to ultrasound criteria established for ADPKD. The criteria included the presence of at least two cysts in one kidney or one cyst in each kidney in an at-risk person aged younger than 30 years, the presence of at least two cysts in each kidney in an at-risk aged between 30 and 59 years, and at least four cysts in each kidney for those persons at risk aged 60 years and older. Furthermore, most affected persons had positive family history of ADPKD. Families with clearly linked disease to the PKD2 according to linkage analysis were excluded.

Blood from 261 (136 males, 125 females) dialyzed pts, or pts who underwent renal transplantation, was sent in EDTA tubes for DNA isolation and G-protein β_3-subunit C825T and endothelial nitric oxide synthase Glu298Asp genotype determination from all cooperating centres. Centres were questioned about the age of ESRF and the presence of hypertension or antihypertensive therapy before ESRF. Pts with ESRF were divided in three groups: 1. 50 pts (22 males, 28 females) with ESRF later than in 63 years (slow progressors), 2. 142 pts (72 males, 70 females) with ESRF between 45–63 years and 3. 73 pts (44 males, 29 females) with ESRF before 45 years (rapid progressors). All pts with ESRF later than in 63 years were analyzed by heteroduplex analysis and sequencing for the PKD2 mutations (data not shown). 4 pts (2 males, 2 females) were further excluded, because the mutation in the PKD2 gene was established.

Moreover, we analyzed 45 ADPKD unrelated pts (17 males, 28 females) in mean age 51.1±8.7 years with serum creatinine under 110 μmol/L (slow progressors) and 200 genetically unrelated healthy Czech subjects (100 males, 100 females, mean age 51.2±8.2).

Methods

Genomic DNA was isolated from peripheral–blood lymphocytes by salting out procedure.[26] G-protein β_3-subunit C825T polymorphism was analyzed by polymerase chain reaction (PCR). The PCR amplification of genomic DNA was followed by restriction digest as recently described by Siffert et al.[27] The following primers were used for PCR: the forward primer 5′-TGACCCACTTGCCACCCGTGC-3′ and the reverse primer 5-GCAGCAGCCAGGGCTGGC-3′. PCR conditions were following: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation, annealing (60°C) and extension. PCR resulted in a 368-bp fragments, which were subsequently restriction-digested with 1U of the enzyme *Bse*D1 for 3 h in 37°C using the buffer recommended by the manufacturer, leading to the generation of a 116-bp and 152-bp fragment with the C allele. The enzyme does not cleave the T allele. Hence,

Table 1. Distribution of G-protein β_3-subunit C825T genotypes among ADPK patients.

	TT	CT	CC
Slow progressors	6.6%	54.9%	38.8%
Rapid progressors	9.6%	46.6%	43.8%
ESRF between 45–63 years	9.2%	50%	40.8%
Control group	12%	44%	44%

CT heterozygotes exhibit all 3 fragments (368, 152 and 116 bp). The fragments were separated on 3% agarose gel with ethidium bromide and visualized under ultraviolet light.

The endothelial nitric oxide synthase Glu298Asp polymorphism was detected by performing PCR amplification followed by restriction digest as described by Miyamoto et al.[23] The following primers were used for PCR: the forward primer 5′-AAGG-CAGGAGACAGTGGATGGA-3′ and the reverse primer 5′-CCCAGTCAATCCCTTTGGTGCTCA-3′. PCR conditions were following: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation, annealing (63°C) and extension. A 248-bp fragment was digested with BanII restriction enzyme in two fragments 158 bp and 90 bp in the presence of thymine at nucleotide position 894 resulting in an aspartic acid at amino acid position 298. The PCR products were visualized by ethidium bromide staining after electrophoresis in 2% agarose gel.

Statistical Analysis

A χ^2-test was used to estimate the frequencies of different genotypes between the groups, assuming Hardy-Weinberg equilibrium. The ages of ESRF between different groups were compared by two-tailed *t*-test. A P value <0.05 was considered significant.

RESULTS

The gender distribution was not significantly different between the selected groups. 90% of pts were hypertensive or used antihypertensive drugs when they reached ESRF. Due to this fact, the evaluation of the relationship between AH and different genotypes could not be accomplished.

Distribution of the G-Protein β_3-Subunit C825T Genotype

The G-protein β_3-subunit C825T genotype exhibited no significant differences among the groups of slow progressors (6.6% (6/91) TT, 54.9% (50/91) CT, 38.8% (35/91) CC), rapid progressors (9.6% (7/73) TT, 46.6% (34/73) CT, 43.8% (32/73) CC), ADPKD group with ESRF between 45–63 years (9.2% (13/142) TT, 50% (71/142) CT, 40.8% (58/142) CC) and control group (12% TT, 44% CT, 44% CC) (Table 1). T-allele frequency was 0.28 in rapid progressors and 0.34 in slow progressors and in APDKD patients with ESRF between 45–63 years. When comparing the ages of ESRF of all patients with ESRF, we did not find significant differences in the ages: males TT—51.7± 8.8 years, CT −51.9±10.3 years, CC −49.7±10.2 years and females TT −56±9.9 years, CT—53.2±8.5 years, CC—53.9±8.7 years.

Distribution of the Endothelial Nitric Oxide Synthase Glu298Asp Genotype

The endothelial nitric oxide synthase Glu298Asp and Asp29Asp genotypes were significantly more frequent in rapid progressors (9.6% (7/73) Asp/Asp, 39.7% (29/73) Asp/Glu, 50.7% (37/73) Glu/Glu) and ADPKD group with ESRF between 45–63 years (11.3% (16/142) Asp/Asp, 41.5% (59/142) Asp/Glu,

Table 2. Distribution of eNOS Glu298Asp genotypes among ADPKD patients.

	Asp/Asp	Asp/Glu	Glu/Glu
Slow progressors	8.8%	24.2%	67%
Rapid progressors	9.6%	39.7%	50.7%
ESRF between 45–63 years	11.3%	41.5%	47.2%
Control group	8%	32%	60%

47.2% (67/142) Glu/Glu) in comparison with slow progressors (8.8% (8/91) Asp/Asp, 24.2% (22/91) Asp/Glu, 67.0% (61/91) Glu/Glu) and control group (8% Asp/Asp, 32% Asp/Glu, 60% Glu/Glu) (Chi-square test, $p<0.05$) (Table 2). Among 73 rapid progressors, there were 44 males (4.5% (2/44) Asp/Asp, 40.9% (18/44) Asp/Glu, 54.6% (24/44) Glu/Glu) and 29 females (17.3% (5/29) Asp/Asp, 37.9% (11/29) Asp/Glu, 44.8% (13/29) Glu/Glu), no significant gender-mediated preferential distribution of Asp variant was noted. Asp variant frequency was 0.29 in rapid progressors, 0.32 in ADPKD patients with ESRF between 45–63 years and 0.21 in slow progressors.

When comparing the ages of ESRF of all patients with ESRF, we did not find significant differences in the ages in males Asp/Asp—54.9±10.4 years, Asp/Glu—50.2±9.4 years, Glu/Glu—51.0±10.4 years. We found out in homozygous Asp/Asp females significantly earlier onset of ESRF (49.2±5.6 years) in comparison with heterozygous females (53.3±7.2 years) and Glu/Glu homozygous females (54.8±9.7 years) (*t*-test, $p<0.05$).

DISCUSSION

No significant genotype distribution differences of G-protein β_3-subunit genotypes distribution or T allele frequencies were established between rapid and slow progressors of the disease in this retrospective study of ADPKD Czech patients. The age differences according to different genotypes were not significant.

The rationale for our study was based on the assumption that the 825T allele to increased activation of G-proteins from immortalized B-cell lines.[9] Increased expression of a splice variant of the $G\beta_3$ subunit (in the presence of T-allele) was identified in cell lines derived from hypertensive individuals on the basis of an increased activity of the Na^+/H^+ enchanter.[28] Furthermore, T-allele was associated with enhanced renal perfusion in early hypertension.[29] We supposed the negative influence of hypertension on the progression of ADPKD. On the other hand, the 825-allele has also been associated with low plasma renin and elevated aldosterone to renin ratio, which could have a protective role in cystogenesis.[30] A functional splice variant in the T-allele carriers causing the intracellular signal transduction via pertussin toxin-sensitive G-proteins and the association of T allele with obesity may accentuate atherosclerotic vascular-renal complications, which can contribute to increased mortality in ADPKD patients and so can lead to the decrease of T allele frequency already before the onset of ESRF. Enhanced signal transduction resulting in increased c-AMP concentration could promote the cystogenesis in the T-allele carriers.

In contrast, the Glu298Asp polymorphism of eNOS was significantly more frequent in ADPKD patients with ERSF earlier than in 45 years (rapid progressors) in comparison with ADPKD patients with ESRF later than in 45 years independently on the gender. We compared together Asp homozygous and heterozygous individuals because of a limited number of homozygous patients for Asp variant.

Asp 298 variant is associated with hypertension in males and females,[22,23] myocardial infarction.[24] ADPKD is associated with an alteration of endothelium-dependent vasodilation, caused by a decreased production of NO by eNOS.[25] A recent study using mice with disrupted eNOS gene revealed that eNOS function is required for vascular and hemodynamic responses to acetylcholine and that the disruption of the eNOS gene leads to hypertension.[31] The whole-body NO production in patients with essential hypertension is diminished under basal conditions, as established by measurement of urinary and plasma nitrate.[32] Despite an identical eNOS enzyme activity, the Asp298 eNOS variant is more vulnerable to enzymatic cleavage in cell lysates compared with the Glu298 protein resulting in lower plasma NO level. Firstly, we supposed the negative influence of hypertension on the progression of ADPKD. Secondly, ADPKD is associated with endothelial dysfunction as in above three mentioned conditions.[25]

Persu et al.[33] confirmed the negative influence 298 Asp variant on the progression of ADPKD in a limited group of males. The influence of eNOS polymorphism in exon 7 was excluded in other study.[34]

The comparison of ESRF ages showed significantly lower age of ESRF in homozygous female with Asp variant of polymorphism. Estrogens regulate the production of NO through eNOS.[35] The menopause and male gender are associated with reduced arterial NO activity. The result could be influenced by a limited number of homozygous patients for Asp variant. The estrogen status of examined females was not known. However, the higher vascular NO activity in premenopausal females could be probably easier influenced by the changes of eNOS activity according to different eNOS genotypes. Moreover, several biochemical pathways can influence NO actions resulting in oxidative stress products, which can contribute to protein and DNA oxidations and promote cell apoptosis.[36] So, higher level of NO in premenopausal females could have some negative effect influenced by many unknown exogenous factors.

In conclusion, we excluded the influence of C825T polymorphism of G-protein beta 3 subunit on the progression of ADPKD in Czech patients. We established negative influence of 298 Asp variant of eNOS polymorphism on the progression of ADPKD. A 5-year lower mean age of ESRF was found in Asp homozygous females in comparison with Glu homozygous females.

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