Original Article

MTHFR and HFE, but Not Preproghrelin and LBP, Polymorphisms as Risk Factors for All-Cause End-Stage Renal Disease Development

(chronic renal failure / end-stage renal disease / haemodialysis / polymorphism / lipopolysaccharide-binding protein / ghrelin / HFE / MTHFR / single-nucleotide polymorphism)

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Abstract. End-stage renal disease (ESRD) is a serious health problem worldwide. The high prevalence of cardiovascular diseases and chronic inflammation remains a major cause of morbidity and mortality in haemodialysed patients. Beside some external factors, genetic predisposition both to renal failure and poor prognosis has been assumed. We have collected a total of 1,014 haemodialysed patients and 2,559 unrelated healthy Caucasians. Single-nucleotide polymorphisms (SNPs) in genes for preproghrelin (GHRL), lipopolysaccharide-binding protein (LBP), HFE and MTHFR were genotyped. In the group of patients, significantly more carriers presented the *MTHFR* T667T (P = 0.002) and *HFE* Asp63Asp (P = 0.001) and Cys282Cys (P = 0.01) genotypes. The frequencies of individual SNPs within *GHRL* and *LBP* genes did not differ between the patients and controls. The trends in genotype frequencies did not differ between the subgroups of patients with different time on haemodialysis. Common variants in *MTHFR* and *HFE* could be a risk factor for all-cause ESRD development, but are not predictors for the survival on haemodialysis.

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Abbreviations: BMI – body mass index, CRP – C-reactive protein, CVD – cardiovascular disease, ESRD – end-stage renal disease, GHRL – ghrelin, HFE – haemochromatosis, HD – haemodialysis, LBP – lipopolysaccharide-binding protein, MTHFR – methylenetetrahydrofolate reductase, PCR-RFLP – polymerase chain reaction-restriction fragment length polymorphism, RBC – red blood cells, SNP – single-nucleotide polymorphism, URR – urea reduction ratio, WBC – white blood cells.

Introduction

Worldwide, the increasing number of individuals in end-stage renal disease (ESRD) represents a serious health, social and economic problem. Although the advances in renal replacement therapy techniques have improved since the last 40 years, cardiovascular disease (CVD) and chronic inflammation still remain the main causes of morbidity and mortality of patients treated with haemodialysis (HD) (Wanner et al., 2002). Since clinical experience has shown large individual differences in manifestation of ESRD and its complications independently from duration of HD therapy, genetic predisposition might be considered. Out of many possible candidate genes, we have selected the variants in genes for lipopolysaccharide binding protein (*LBP*), haemochromatosis (*HFE*), methylenetetrahydrofolate reductase (*MTHFR*) and ghrelin (*GHRL*) for analysis.

ESRD, usually associated with uraemia, is a chronic pro-inflammatory immunosuppressive condition characterized both by markers of suppression and activation of the immune system (Macdougall, 2004). The disturbances of the immune system involve defective both innate (reduced chemotaxis, phagocytosis, bactericidal activity) and acquired (reduced T-cell and antigen-presenting cell activity) immunity with excessive production of pro-inflammatory cytokines even in the absence of clinical infection that contributes to the generalized immunodeficiency (Kalousová et al., 2003; Lim et al., 2007). The infectious complications are just the second leading cause of death in HD patients (Eleftheriadis et al., 2007). For the adequate immune response to gram-negative infection, LBP is fundamental for lipopolysaccharide-dependent monocyte response adjustment. The LBP plasma levels have shown to be significantly higher in patients with chronic renal failure than in healthy controls (Pereira et al., 1996). Because of the correlation of plasma LBP and C-reactive protein (CRP) levels, the *LBP* gene is a good candidate for determination of inflammatory state in ESRD (Pavcnik-Arnol et al., 2004).

The chronic inflammation aggravates the already present metabolic disturbances resulting in malnutrition

characterized by depleted energy stores (loss of fat tissue and somatic proteins) and decrease of body weight. The nutritional state in HD population has a complex background involving hypercatabolism in general, insufficient protein-caloric intake, defective food assimilation and utilization, uraemia (appetite loss) and HD treatment (loss of amino acids and proteins) (Basile, 2003). Ghrelin (GHRL), an endogenous ligand for growth hormone secretagogue receptor, regulates food intake and energy balance through a direct effect on hypothalamus (Gardiner et al., 2008). Three genetic polymorphisms with presumable connection with obesity have been identified so far (Ukkola et al., 2002; Vivenza et al., 2004). In HD patients, the ghrelin plasma levels are elevated compared to healthy individuals and correlate with their nutritional state (Perez-Fontan et al., 2004; Chang et al., 2005; Jarkovská et al., 2005). It has also been suggested that carriers of the Gln51 allele exhibit lower and Met72Met homozygotes elevated plasma ghrelin concentration in comparison to non-carriers (Ukkola et al., 2002).

Chronic anaemia, commonly present in HD patients and caused by persistent cytokine synthesis with impairment of intracellular iron metabolism and inhibition of erythroid progenitor cells in bone marrow, correlates with worse prognosis (Stenvinkel, 2001). Mutations in the *HFE* gene resulting in defective interaction with β_2 microglobulin essential for iron metabolism have recently been shown to be responsible for hereditary haemochromatosis, and thus may be beneficial in patients on haemodialysis while improving their iron availability (Canavesi et al., 2012). The direct atherogenic properties of secreted pro-inflammatory cytokines affect endothelial dysfunction that together with slightly impaired folate metabolism due to *MTHFR* polymorphism could result in atherosclerosis acceleration and high prevalence of cardiovascular disease in haemodialysis patients (Kimura et al., 2000; Juo et al., 2008). In the present study we have concentrated on possible genetic predispositions associated with ESRD and prognosis.

Material and Methods

Subjects

For study involvement, the patients had to fulfil the following criteria: HD therapy for three months at least, absence of generalized cancer disease, three years of survival expectance, agreement with participation.

One thousand-fourteen patients (~ 25 % of Czech HD population) were enrolled into the study. In case of death (N = 108) or kidney transplantation (N = 78) the patient was excluded from the prospective part of the study. This prospective follow-up by means of specially created electronic questionnaire containing clinical (cause of ESRD, parameters of HD therapy, presence of diabetes, cardiovascular disease, hepatitis) and laboratory (CRP, creatinine, urea, lipids, BMI, albumin, WBC, RBC, haemoglobin, haematocrit, transferrin, iron, URR, CaP product) parameters was finally performed in 607 patients only (60 %) (Table 1). The primary measurements were made at study enrolment. Because of heterogeneity, for more detailed analysis the ESRD population was divided into three subgroups depending on the duration of HD therapy with previous exclusion of transplanted (N = 69) and deaths (N = 92) within 2–7 years on HD, into one-year HD survivors (N = 421), eight-year HD survivors (N = 137) and deaths within a year on HD (N = 49).

The control population without renal disorders consisted of 2,559 unrelated Caucasians (1,191 males and 1,368 females, aged 28–67 years) representing a 3-year cohort of the selected 1 % Czech population sample (Multinational monitoring of trends and determinants in cardiovascular diseases: MONICA Project).

The study was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine.

Table 1. Basic characteristics of the 607 individuals with 3-month follow-up

	Deaths within 1st year on HD	**One-year HD survivors**	**Eight-year HD survivors**
N	**49**	**421**	**137**
Age (years)	69.9 ± 12.0	66.1 ± 12.9	64.7 ± 12.9
CVD (%)	20.4	22.8	14.6
BMI (kg/m^2)	25.2 ± 7.1	26.9 ± 5.4	24.9 ± 4.3
Cholesterol (mmol/l)	4.08 ± 0.93	4.71 ± 1.06	4.73 ± 0.89
Triglycerides (mmol/l)	1.63 ± 0.97	2.36 ± 1.65	1.69 ± 0.81
Albumin (g/l)	36.5 ± 5.3	39.7 ± 4.1	39.4 ± 4.0
Urea (mmol/l)	18.6 ± 5.8	20.7 ± 6.2	21.3 ± 6.1
CRP (mg/l)	41.0 ± 55.7	13.7 ± 18.7	14.7 ± 18.8
WBC (10^9/l)	8.43 ± 2.67	7.27 ± 2.19	6.89 ± 2.20
RBC (10^{12}/l)	3.47 ± 0.49	3.57 ± 0.51	3.76 ± 0.54
Haemoglobin (g/l)	103.8 ± 25.3	106.7 ± 26.3	117.9 ± 20.2
Haematocrit	0.34 ± 0.05	0.35 ± 0.05	0.37 ± 0.05
Transferrin (mmol/l)	1.59 ± 0.29	2.00 ± 0.69	1.93 ± 0.66
Fe (μmol/l)	13.09 ± 8.49	13.02 ± 6.26	13.87 ± 7.52

DNA extraction and analysis

DNA was extracted from 5 ml of whole uncoagulated (EDTA) blood samples by the standard salting-out method (Miller et al., 1988). DNA variants (all single-nucleotide polymorphisms – SNPs) of *GHRL* (Arg51Gln, Leu72Met and Gln90Leu), *LBP* (T291C and Leu436Phe), *HFE* (Asp63His and Cys282Tyr) and *MTHFR* (C667T/Ala236Val) genes were analysed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)-based methods in accordance with procedures presented in detail previously (Hubacek et al., 2001, 2007; Moriyama et al., 2002; Barber and O'Keefe, 2003; Cimburová et al., 2005).

Statistical analysis

Statistical analysis was performed using the Fisher's exact test and ANOVA. In case that there were less than five individuals carrying one genotype (at least in controls or patients), for the analysis they were pooled with heterozygotes. Values are given as means ± SD. Due to multiple testing, a P value = 0.01 was considered to be significant.

Results

The genotype call rates for individual genotypes were between 90.2 % and 97.8 % in the patients and between 94.8 % and 99.9 % for healthy controls. In both groups, the lowest call rate was obtained for the Cys282Tyr *HFE* variant.

In the acquired sampling of HD patients, the most common verified cause of ESRD was diabetic nephropathy, tubulointerstitial nephropathy and nephrosclerosis based on hypertension. Unfortunately, in the largest part of HD patients (~ 40 %) the renal biopsy was performed in the phase of end-stage kidney and, therefore, they could not be divided according to primary renal disease.

The individuals who died within the first year of HD therapy exhibited significantly lower dry body weight (P = 0.0023), lower levels of albumin (P = 0.0034), RBC (P = 0.0072), haemoglobin (P = 0.005), haematocrit (P = 0.004) and higher level of WBC (P = 0.0199) compared to both HD groups (Table 1). The HD groups did not differ in evolution of laboratory data in three years of follow-up. The genotype frequencies of the analysed polymorphisms of *GHRL, LBP, HFE* and *MTHFR* genes are summarized in Table 2. In the control group, the genotype distribution was in Hardy-Weinberg equilibrium in all cases (P values between 0.15 and 0.91). In HD patients, the variants for *GHRL* (Arg51Gln, the entire population and both males and females; Gln90Leu, males only) and for *HFE* (Asp63His, the entire population and males) were not within Hardy-Weinberg equilibrium. This was mainly caused by higher frequency of rare homozygotes – these individuals were twice re-genotyped to avoid miss-genotyping, with the same results. Comparing the genotype frequencies between ESRD and healthy populations, we observed significant differences in *MTHFR* and *HFE* gene variants, but not in *GHRL* or *LBP*.

We found significant differences in the frequencies of the *MTHFR* C667T (P < 0.002 for the entire group, P = 0.039 for males, P = 0.043 for females), *HFE* Asp63His (P = 0.01 for the entire group, P = 0.037 for males, P = 0.421 for females) and finally Cys282Tyr genotypes (P = 0.001 for the entire group, P = 0.055 for males, P = 0.006 for females) between the HD patients and healthy controls (Table 2). When the HD patient subgroups were compared, there were no significant differences in genotype distribution depending on duration of the HD therapy (Table 3).

Discussion

The exact pathogenesis of chronic renal failure has not yet been sufficiently understood. Traditional risk factors such as age, black race, male gender, smoking, hypertension, hyperlipidaemia, diabetes and obesity cannot explain the complete individual susceptibility to development of ESRD and accompanying complications that essentially determine the clinical outcome. Because of clinically experienced inter- and intra-individual differences, an important role of genetic predisposition might be assumed.

For example, the variants in *MTHFR, ACE, APOE, TGFb, TNFa, IL6* and *10, GST, TRPC6, FTO* and *END1* genes were analysed in patients with renal disease (Agrawal et al., 2007; Arikan et al., 2007; Balakrishnan and Rao, 2007; Girndt et al., 2007; Maixnerová et al., 2007; Bloudíčková et al., 2011; Hubacek et al., 2012; Obeidova et al., 2012). The conflicting results of present studies are given by evaluation of distinct ethnic groups, differences in primary renal disease leading to ESRD, or low rate of included individuals causing false--positive results.

This study was performed to examine the variants of candidate genes with possible relationship to the development of ESRD and complications of HD therapy that have not been analysed so far. Concentrating on the differences in genotypes between extensive heterogeneous HD and healthy populations, our results support the *MTHFR* C667C homozygosity as one of the causative factors involved in renal damage and end-stage renal disease (Table 2). Many studies have previously shown conflicting data concerning the role of *MTHFR* T667C variant in the pathogenesis of kidney disease – some researchers have demonstrated an association but others have not (Zychma et al., 2002; Tylicki et al., 2005). Because of higher incidence of *HFE* His63His and Cys282Cys homozygotes in HD population, we suggest that these polymorphisms could have an unfavourable impact on ESRD development. However, in this case we have to be careful with the definitive conclusion as the Asp63His variant in male patients was out of the Hardy-Weinberg equilibrium; however, this discrepancy could in fact be caused by the disease. In HD patients, the *GHRL* and *LBP* genes have not been studied

Table 2. Genotype frequencies of analysed genes in haemodialysed patients and controls. P values for each SNP are given for the comparison between the entire populations, males and females

Gene	Control population				HD patients			
	Males		Females		Males		Females	
	N	%	N	%	N	%	N	%
Ghrelin								
Arg51Arg	1134	95.3	1301	95.2	515	93.8	419	95.6
Gln51Arg	56	4.7	64	4.7	31	5.7	17	3.9
Gln51Gln	0	0	2	0.1	3	0.5	2	0.5
P				0.223	0.193	0.673		
Leu72Leu	1021	85.9	1168	85.6	495	85.9	345	85.5
Leu72Met	164	13.8	189	13.9	71	13.0	62	14.3
Met72Met	4	0.3	7	0.5	6	1.1	1	0.2
P							0.983	0.740	0.591
Gln90Gln	1052	88.6	1219	89.4	491	88.8	378	86.9
Gln90Leu	129	10.9	137	10.1	61	11.0	51	11.7
Leu90Leu	6	0.5	7	0.5	1	0.2	6	1.4
P				0.351	0.921	0.143		
LBP								
T291T	847	71.2	959	70.2	385	69.6	294	68.4
T291C	314	26.4	370	27.1	150	27.1	119	27.7
C291C	28	2.4	37	2.7	18	3.3	17	4.0
P				0.227	0.504	0.389		
Leu436Leu	962	80.8	1098	80.3	439	79.8	329	77.4
Leu463Phe	215	18.1	254	18.6	104	18.9	93	21.9
Phe436Phe	13	1.1	16	1.2	7	1.3	3	0.7
P				0.437	0.858	0.241		
HFE								
Asp63Asp	875	73.4	1000	73.4	388	69.6	327	75.4
His63Asp	292	24.5	337	24.7	142	25.7	96	22.1
His63His	25	2.1	26	1.9	23	4.7	11	2.5
P							0.01	0.037	0.421
Cys282Cys	1026	94.6	1154	94.4	503	92.1	390	90.5
Cys282Tyr	58	5.3	66	5.4	43	7.9	40	9.3
Tyr282Tyr	1	0.1	2	0.2	0	0	1	0.2
P				0.001	0.055	0.005		
MTHFR								
C667C	515	43.3	577	42.2	272	48.4	202	47.0
C667T	527	44.3	628	46.0	240	42.7	194	45.1
T667T	147	12.4	161	11.8	50	8.9	34	7.9
P				0.002	0.039	0.043		

so far, and we did not associate them with ESRD development. No prospective study has been performed with a representative sample of patients in ESRD treated with HD yet comparable to studies dealing e.g. with cardiovascular diseases (Nordfors et al., 2005). The observed laboratory data in the individuals who died within a year of HD therapy is also in accordance with their poor clinical outcome compared to one- and eight-year HD therapy survivors.

In our present study, the SNPs in methylenetetrahydrofolate reductase and haemochromatosis genes were found to be associated with the development of all-cause ESRD, but not with the duration of HD therapy.

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Table 3. Genotypes of analysed genes and effect on the patients' survival on haemodialysis

	Deaths within 1st year on HD (N/%)		One-year HD survivors (N/%)		Eight-year HD survivors (N/%)		
	N	%	N	%	N	%	
Ghrelin							
Arg51Arg	44	91.7	386	93.7	130	95.6	0.06
Gln51Arg	4	8.3	25	6.1	6	4.4	
Gln51Gln	0	0	1	0.2	0	0	
Leu72Leu	43	89.6	341	83	116	84.7	0.48
Leu72Met	5	10.4	65	15.8	21	15.3	
Met72Met	0	0	5	1.2	0	0	
Gln90Gln	38	80.9	365	88.4	115	83.3	0.22
Gln90Leu	8	19.1	47	11.4	21	15.2	
Leu90Leu	0	0	1	0.2	2	1.5	
LBP							
T291T	31	72.1	271	68.3	96	73.3	0.52
T291C	9	20.9	114	28.7	27	20.6	
C291C	3	7.0	12	3.0	8	6.4	
Leu436Leu	39	83.0	306	74.6	115	84.6	0.04
Leu436Phe	8	17.0	100	24.4	19	14.0	
Phe436Phe	0	0	4	1	2	1.4	
HFE							
Asp63Asp	32	66.7	295	71.6	97	71.3	0.77
Asp63His	11	22.9	107	26	32	23.5	
His63His	5	10.4	10	2.4	7	5.2	
Cys282Cys	39	92.9	327	89.8	121	95.3	0.16
Cys282Tyr	3	7.1	36	9.9	6	4.7	
Tyr282Tyr	0	0	1	0.3	0	0	
MTHFR							
C667C	24	51.1	207	50.1	67	48.9	0.93
C667T	19	40.3	177	42.9	60	43.8	
T667T	5	10.6	29	7.0	10	7.3	

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