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Retinopathy and Nephropathy in Type 1 Diabetic Patients – Association with Polymorphysms of Vitamin D-Receptor, Tnf, Neuro-D and Il-1 Receptor 1 Genes

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A B S T R A C T

Retinopathy and nephropathy are common late type 1 diabetes mellitus (T1D) complications. In this study we investigated whether individual differences in 4 candidate genes significantly contribute to development and progression of late complications in T1D patients. We examined 121 patients for the presence of diabetic retinopathy and nephropathy. We genotyped variants in vitamin D receptor (VDR) and tumor necrosis factor (TNF) genes in 47 patients and in NeuroD1 and interleukin-1 receptor 1 (IL1R1) genes in 35 patients. Diabetic retinopathy had 66 (55%) patients after a median of 13.0 years after diagnosis. Diabetic nephropathy had 14 (11.66%) patients, all of whom had already developed retinopathy. A significant correlation between the degree of diabetic retinopathy and mean microalbuminuria (MA) value has been found (χ^2=54.18, p<0.001). After correcting for duration of disease, only the VDR gene BsmI genotypes showed significant association with cumulative prevalence of diabetic retinopathy, while no investigated genetic polymorphysms could reliably predict diabetic nephropathy.

Key words: *diabetes mellitus Type 1, genes, diabetic retinopathy, diabetic nephropathy, microalbuminuria, Croatia*

Introduction

Diabetes mellitus comprises several different clinical syndromes, which have in common chronic hyperglycemia and metabolic disorders, which later lead to ocular, renal, neuronal and vascular complications[1]. Microangiopathy, as a chronic diabetic complication, involves particularly small retinal, neuronal, and glomerular vessels[2]. Epidemiological data show that diabetic retinopathy and nephropathy are closely correlated. About 90% of patients with a history of more than 20 years of type 1 diabetes mellitus (T1D) develop diabetic retinopathy (15–20% develop blindness) and 25–40% develop diabetic nephropathy[3,4]. Early onset of diabetic retinopathy is often associated with nephropathy, although the mechanism is still not fully understood[5]. However, other authors described cases of quite advanced renal dysfunction with modest or even inexistent retinal signs of diabetic retinopathy[6,7]. T1D is complex disease and has a strong genetic component[8]. Vitamin D, tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1 may play a critical role in the pathogenesis of inflammatory and autoimmune disease like T1D[9–11]. All of them are involved in immune reactions in the organism. Vitamin D has immunosuppressive effect exerted via promotion of monocyte differentiation and inhibition of lymphocyte proliferation and secretion of cytokines, such as IL-2, interferon-γ and IL-12[9]. TNF-α can be cytotoxic for β-cells supported by both IL-1 and interferon-γ[10]. IL-1 in very low concentrations has cytotoxic effects on islets of Langerhans[11].

The effects of vitamin D are mediated by a nuclear receptor (VDR) that acts as a ligand-activated transcrip-

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tion factor[12]. The VDR gene located on chromosome 2q14, has several frequently studied single nucleotide polymorphisms (SNP). These are FokI G/A change in exon 2, TaqI T/C change in exon 9, BsmI A/G, Tru9I G/A and ApaI G/T changes in intron 8[13]. FokI occurs at the first start codon in exon 2 and changes the translation initiation site resulting in a three amino acids shorter protein which is considered to be more active than the wild-type[14].

TNF-α is coded by TNF gene located on chromosome 6 in the HLA region class III[15]. Two promoter SNPs at positions –308 G/A and –238 G/A of the TNF gene have been commonly studied. The polymorphisms within the TNF promoter region may affect transcription factor binding sites resulting in TNF-α differential expression, which can modulate the risk to T1D[16]. IL-1 type 1 receptor (IL1R1) is found on most cells including T lymphocytes and β cells[17]. IL1R1 gene maps to chromosome 2 (2q12-22)[18]. Several IL1R1 gene SNPs like HinfI G/A, PstI G/A, AluI T/C in the promoter region and PstI-e C/T in exon 1B have been frequently studied[19–21].

Human NeuroD1 gene (known as BETA2 or NeuroD/BETA2) has been mapped on the chromosome 2 (2q32). NeuroD1 is a transcription factor expressed in pancreatic endocrine, intestine and brain cells[22]. It activates the transcription of the insulin gene in pancreatic β cells[22]. It is known that MwoI G/A polymorphism, located in codon 45 of the exon 2 of the NeuroD1 gene, leads to Ala/Thr substitution of protein[23].

The aim of this study was to investigate the association between diabetic retinopathy and nephropathy (incipient and clinical) in patients with T1D and to attempt to predict the development of those complications by associating them to the variants in the recently described candidate genes – VDR, TNF, NeuroD1 and IL1R1.

Materials and Methods

A total of 141 patients with T1D were included in the present study. Nine patients were excluded because they had argon laser photocoagulation previously done, 3 because of terminal renal insufficiency, 4 had urinal tract infection and 5 had poor metabolic disorder regulation (HbA_{1c} >9%) (Figure 1).

Among 120 patients that remained in the study, 65 (54.2%) were men and 55 (45.8%) women. The mean age of onset of diabetes type I was 17.2±9.3 years, and mean duration of the illness was 13.9±9.1. In the study group the mean age was 32.2±13.9 years for men, and 29.2±13.8 years for women, with the median of 13 years of follow-up after diagnosis.

Labotarotory exams included glycosylated hemoglobin (HbA_{1c}-assay used on the Dimension clinical chemistry system) and creatinine clearance. These parameters were used only as orientation of metabolic regulation and renal status and were not used for other analyses performed in this study. To assess the development of retinopathy, all patients underwent through ophthalmo-

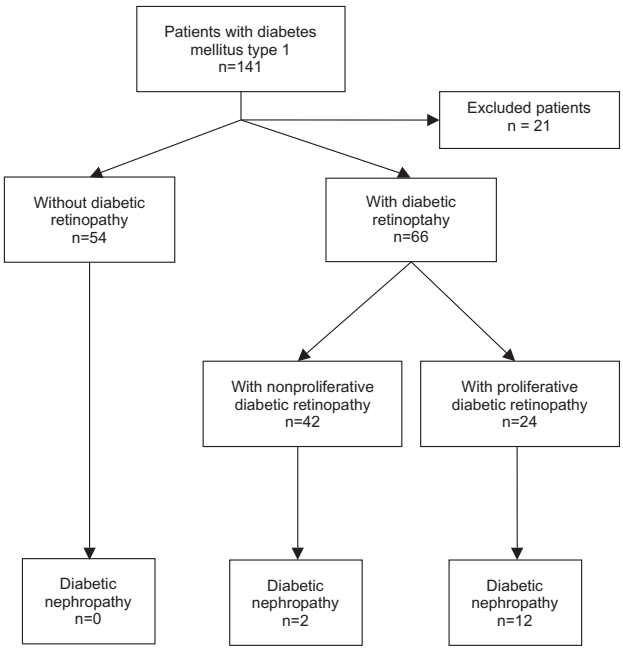

Fig. 1. Flowchart of patients included in the study.

logical examination that included direct and indirect fundus examination with pupils previously dilated (tropicamide 1%) and 30° color fundus single-field photography (fundus camera Zeiss FF 450 plus IR-Germany). If any diabetic changes were detected, data were collected from the examination of the eye with the worse results. According to ophthalmological findings all patients were grouped into three different groups: (i) group without diabetic retinopathy, (ii) group with nonproliferative diabetic retinopathy and (iii) group with proliferative diabetic retinopathy.

To assess the development of diabetic nephropathy (in patient without signs of urinal tract infection from urine sediment examination) the mircroalbuminuria (MA) and the proteinuria was tested by turbidimetric method (ARCHITECT cSYSTEMS – Abbot Diagnostics, USA) twice during 15 days. We used MULTIGENT immunoassay for the quantization of albumins and U-Pro assay for the quantization of proteins. The mean result value ≥30 mg/24 hour was considered as a sign of MA (incipient nephropathy) and ≥0.5 g/24 hours as a sign of proteinuria (clinical or evident nephropathy). Patients were divided according to turbidimetric immunoassay results in three groups: (i) no MA (albumin <30 mg/24h urine), (ii) MA (albumin ≥30–300 mg/24h urine) and (iii) clinical albuminuria (>300 mg/24h urine). According to turbidimetric U-Pro assay results patients were also subdivided in two groups: without diabetic nephropathy (proteinuria<0.5 g/24 hours) and with diabetic nephropathy (proteinuria ≥0.5 g/24 hours).

We have genotyped VDR and TNF candidate genes in 47 patients and NeuroD1 and IL1R1 genes in 35 patients. Genomic DNA was extracted from peripheral blood leucocytes using the Perfect gDNA kit (Eppendorf,

Hamburg, Germany). Genotypes for five gene SNPs in VDR gene (BsmI, ApaI, TaqI, FokI and Tru9I), two gene SNPs in TNF gene (–308 and –238), four gene SNPs in IL1R1 gene (PstI, HinfI, AluI, and PstI-e) and one gene SNP in NeuroD1 gene (MwoI) were identified by PCR followed by RFLP analysis, according to previous reports[14,19,26–29]. Genotypes for the polymorphisms in the VDR and IL1R1 genes were designated with a capital letter for the absence of a restriction site and with a lowercase letter for its presence. Therefore, the genotypes are as follows: BsmI=BB, bb (homozygous for the absence or presence of the cut site, respectively) and Bb (heterozygous); ApaI=AA, aa, Aa; TaqI=TT, tt, Tt; FokI=FF, ff, Ff; Tru9I=UU, uu, Uu; PstI=PP, pp, Pp; HinfI=HH, hh, Hh; AluI=AA, aa, Aa, PstI-e=P'P', p'p', P'p'. Genotypes for the TNF gene and NeuroD1 gene SNPs were designated with GG for guanine homozygotes, AA for adenine homozygotes and AG for adenine/guanine heterozygotes.

The aim was to correlate different variants to progression of complications in T1D and to determine »genotype relative risks« for development of complications of T1D.

Statistical analysis was done with SPSS/PC+ program. ANOVA, χ^2-test, Duncan test and Man-Whitney U-test were used to compare data.

Results

Diabetic retinopathy was found in 66 (55%) patients. Forty two (63.63%) had nonproliferative and 24 (36.37%) had proliferative diabetic retinopathy (Figure 1).

MA was absent in 78 (65%) patients, present in 28 (23%) patients and persistent in 14 (12%) patients. Turbidimetric immunoassay results in different diabetic retinopathy groups are shown in Table 1. There was statistically significant correlation between MA and the degree of diabetic retinopathy (χ^2=54.18, p<0.001). Greater degree of diabetic retinopathy is correlated with higher incidence of MA and vice versa. Particularly high positive correlation was found in group with proliferative diabetic retinopathy.

TABLE 1
DISTRIBUTION OF PATIENTS ACCORDING TO GRADE OF DIABETIC RETINOPATHY AND MICROALBUMINURIA

	N°of patients with microalbuminuria			Total
	<30 mg/24h	≥30–300 mg/24h	>300 mg/24h	
Without diabetic retinopathy	47	7	0	54
Nonproliferattive diabetic retinopathy	26	14	2	42
Proliferative diabetic retinopathy	5	7	12	24
Total	78	28	14	120

MA mean value in the group of patients without diabetic retinopathy was 23.5±44,6 mg/24h urine, 68.9±117.9 mg/24h urine in the group with nonproliferative diabetic retinopathy and 899.1±1300 mg/24h urine in the group of patients with proliferative diabetic retinopathy. There was statistically significant difference in MA mean values between three diabetic retinopathy groups (χ^2=39.5, p<0.001). Patients with normal fundus findings had normal MA mean value, while in more advanced stages of diabetic retinopathy MA mean value was higher.

In the group of patients without diabetic retinopathy there were no patients with diabetic nephropathy, while 2 (4.8%) patients in the group with nonproliferative diabetic retinopathy and 12 (50%) patients in the group with proliferative diabetic retinopathy had diabetic nephropathy. There was statistically significant correlation of diabetic nephropathy with grade of diabetic retinopathy (χ^2=38.72, p<0.001). Diabetic nephropathy was most commonly found in patients with proliferative diabetic retinopathy.

Proteinuria mean value in the group of patients without diabetic retinopathy was 0.08±0.09 g/24h urine, 0.16±0.21 g/24h urine in the group with nonproliferative diabetic retinopathy and 1.41±2.0 g/24h urine in the group of patients with proliferative diabetic retinopathy. There was statistically significant difference in proteinuria mean values between three diabetic retinopathy groups (χ^2= 27.34, p<0.001). Greatest mean value of proteinuria was found in patients with proliferative diabetic retinopathy.

The longer the duration of T1D, the higher the probability of developing higher grade diabetic retinopathy, because the mean duration of T1D was 7.4±5.6 years in the group without diabetic retinopathy, 17.4±7.9 years in the group with nonproliferative diabetic retinopathy, and 22.4±6.8 years in the group with proliferative diabetic nephropathy (F=49.84, p<0.001).

Predictably, cumulative prevalence of MA increases with the duration of T1D. In the group of patients with no MA, mean duration was 11.2±8.3 years, in the group with MA it was 17.0±8.9 and in the group with macroalbuminuria was 21.0±6.5 years. Analysis of variance showed statistically significant difference in diabetes duration among three groups (F=13.81, p<0.001) (Figure 2). There was also statistically significant difference in diabetes duration between the group with diabetic ne-

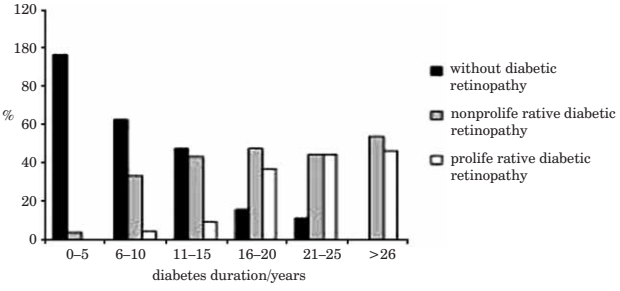

Fig. 2. Cumulative prevalence of various types of diabetic retinopathy according to the duration of diabetes type 1.

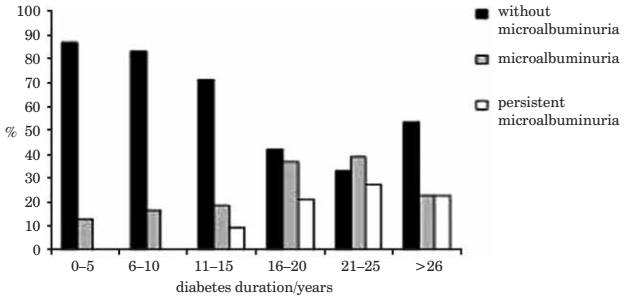

Fig. 3. The incidence of microalbuminuria according to the duration of diabetes type 1.

phropathy (21.4±6.0 years) and the group without diabetic nephropathy (12.8±8.9 years) (t=4.89, p<0.001). (Figure 3).

These relationships between duration of T1D and cumulative prevalence of complications, shown in figures 2 and 3, were very helpful as they were used to standardize the subsequent cumulative prevalence according to different genotypes by duration of disease. Table 2 shows that after correcting for duration of disease, only the BsmI VDR genotypes showed significant association with cumulative prevalence of retinopathy (p<0.05), while no investigated genetic polymorphysms could reliably predict nephropathy as none of the observed differences formally approached statistical significance.

Discussion

Diabetic retinopathy is the most common and most difficult eye complication of diabetes and is the leading cause of blindness in the active population[4]. Ophthalmologist has the possibility of direct examination of the blood vessels, which permits him not only to treat the eye but also to participate actively in prevention, diagnostics and treatment of late diabetic complications. Detection of grades that are still reversible is of crucial importance, because appropriate therapy can significantly postpone or even prevent the onset of irreversible diabetic renal changes. We used fundoscopy and turbidimetric method as most common diagnostic methods, even though much sophisticated methods (as fluorescein angiography and renal biopsy) for determination of diabetic retinopathy and nephropathy are now available. We considered fundoscopy as relevant because some authors have confirmed that 85% of findings obtained by direct or indirect fundoscopy on dilated pupil were the same as those obtained by fundus multi-field photography[30]. In support, we found diabetic retinopathy in 55.0% of patients and diabetic nephropathy in 10.8% of patients, which is near to results found in one previous study where 64.3% of patients had diabetic retinopathy and 14.2% diabetic nephropathy with the similar duration of T1D[31].

Our data show that there is statistical correlation of MA with diabetic retinopathy grade. There was 50%

TABLE 2
EFFECTS OF DIFFERENT GENOTYPES IN POLYMORPHYC REGIONS OF THE FOUR CANDIDATE GENES ON CUMULATIVE PREVALENCE OF COMPLICATIONS OF T1D

Observed genotypes	N (%)	Median duration of DM1	Cumulative prevalence of retinopathy	Cumulative prevalence of nephropathy	p-value: *retinopathy **nephropathy
1. VDR-FokI					
FF	13 (27.7%)	9 mths	6/13 (46.2%)	4/13 (30.8%)	*N.S.
Ff	24 (51.1%)	8.5 mths	9/24 (37.5%)	6/24 (25.0%)	**N.S.
ff	10 (21.3%)	6 mths	4/10 (40.0%)	4/10 (40.0%)	
Total	47 (100%)				
2. VDR-TaqI					
TT	18 (38.3%)	9.5 mths	7/18 (38.9%)	5/18 (27.8%)	*N.S.
Tt	20 (42.6%)	8 mths	8/20 (40.0%)	6/20 (30.0%)	**N.S.
tt	9 (19.1%)	6 mths	4/9 (44.4%)	3/9 (33.3%)	
Total	47 (100%)				
3. VDR-BsmI					
BB	7 (14.9%)	7 mths	2/7 (28.6%)	1/7 (14.3%)	<0.05
Bb	26 (55.3%)	8 mths	10/26 (38.5%)	8/26 (30.8%)	**N.S.
bb	14 (29.8%)	9 mths	7/14 (50.0%)	5/14 (35.8%)	
Total	47 (100%)				
4. VDR-Tru9I					
UU	34 (72.3%)	9 mths	15/34 (44.1%)	12/34 (35.3%)	*N.S.
Uu	12 (25.5%)	8 mths	3/12 (25.0%)	2/12 (16.7%)	**N.S.
uu	1 (2.1%)	7 mths	1/1 (100%)	0/1 (0%)	
Total	47 (100%)				

5. TNF-238I					
AA	0 (0%)	–	–	–	*N.S.
AG	4 (8.5%)	6.5 mths	2/4 (50.0%)	1/4 (25.0%)	**N.S.
GG	43 (91.5%)	9 mths	17/43 (39.5%)	13/43 (30.23%)	
Total	47 (100%)				
6. TNF-308I					
AA	4 (8.5%)	7 mths	1/4 (25.0%)	1/4 (25.0%)	*N.S.
AG	12 (25.6%)	10 mths	7/12 (58.3%)	4/12 (33.3%)	**N.S.
GG	31 (66.0%)	8 mths	11/31 (35.5%)	9/31 (29.0%)	
Total	47 (100%)				
7. NeuroD1					
AA	7 (20.0%)	7 mths	2/7 (28.6%)	1/7 (14.3%)	*N.S.
AG	18 (51.4%)	10 mths	10/18 (55.6%)	7/18 (38.9%)	**N.S.
GG	10 (28.6%)	11 mths	3/10 (30.0%)	3/10 (30.0%)	
Total	35 (100%)				
8. IL1R1-PstI					
pp	1 (2.9%)	13 mths	0/1 (0%)	0/1 (0%)	*N.S.
Pp	15 (42.9%)	9 mths	9/15 (60.0%)	6/15 (40.0%)	**N.S.
PP	19 (54.3%)	10 mths	6/19 (31.6%)	5/19 (26.3%)	
Total	35 (100%)				
9. IL1R1-HinfI					
hh	1 (2.9%)	13 mths	0/1 (0%)	0/1 (0%)	*N.S.
Hh	15 (42.9%)	9 mths	9/15 (60.0%)	6/15 (40.0%)	**N.S.
HH	19 (54.3%)	10 mths	6/19 (31.6%)	5/19 (26.3%)	
Total	35 (100%)				
10. IL1R1-AluI					
aa	6 (17.1%)	11 mths	2/6 (33.3%)	2/6 (33.3%)	*N.S.
Aa	23 (65.7%)	10 mths	11/23 (47.8%)	8/23 (34.8%)	**N.S.
AA	6 (17.1%)	7.5 mths	2/6 (33.3%)	1/6 (16.7%)	
Total	35 (100%)				
11. IL1R1-Pst-e					
P'P'	4 (11.4%)	9.5 mths	2/4 (50.0%)	1/4 (25.0%)	*N.S.
P'p'	21 (60.0%)	8 mths	9/21 (42.9%)	7/21 (33.3%)	**N.S.
P'p'	10 (28.6%)	13 mths	4/10 (40.0%)	3/10 (30.0%)	
Total	35 (100%)				

probability that patient in the group with proliferative diabetic retinopathy had persistent MA and 79% probability of having MA. Furthermore, patients without diabetic retinopathy did not have MA in 24 hour urine and the difference in MA between patients with and without diabetic retinopathy was statistically significant. In our study, diabetic nephropathy was commonly found in patients with proliferative diabetic retinopathy, while was extremely rare in patients without diabetic retinopathy. This is in concordance with some previous studies where patients with T1D and proliferative diabetic retinopathy had five times grater risk of having proteinuria[32]. This underscores the importance of turbidimetric test in 24 hour urine, especially in patients who have developed diabetic retinopathy. For example, in the present study, in case of 14 patients with nonproliferative diabetic retinopathy and 7 patients with proliferative diabetic retinopathy, early detection of incipient diabetic nephropathy would be done by turbodimetric imunoassay and consequently would get an adequate and timely treatment. There was an obvious direct correlation between diabetic nephropathy and proliferative diabetic retinopathy, what was also reported in WESDR study[33].

After correcting for duration of disease, only the BsmI VDR genotypes showed significant association with cumulative prevalence of retinopathy in the patients of South Croatia. In the two separate studies of the VDR gene in the French population, FokI and TaqI SNPs associations with diabetic retinopathy have been suggested[34,35]. However, Capoluongo et al did not find FokI and BsmI SNPs associations with diabetes complications in the Italian population[36].

The duration of T1D is in strong correlation with development of diabetic nephropathy. In more recent studies it was found that 30–45% microalbuminuric patients progress to proteinuria over 10 years[25]. Understanding that the mean time of diabetes duration in mentioned groups does not mean coincident onset of functional disorders, that time lap implies the need for MA testing in order to start the adequate treatment as soon as possible. Similarly, significant difference in T1D duration was found among groups with various grade of diabetic retinopathy. We have found nonproliferative diabetic retinopathy in only 1 (4%) patient among 25 patients with diabetes duration up to 5 years, while other authors found 0–6% of their patients to have developed diabetic retinopathy within first five years[31,37–39]. Proliferative diabetic retinopathy is rare until 5 years diabetes duration, and afterwards it begins to be more frequent. The 4-year incidence of developing proliferative retinopathy in the WESDR younger-onset group increased from 0% during the first 5 years to 27.9% during years 13–14 of diabetes. After 15 years, the incidence of developing proliferative diabetic retinopathy remained stable[38,40,41]. We had 42% of patients with proliferative diabetic retinopathy in the group of patients with over 15 years of diabetes duration, while Eriksen et al. reported 60%[42]. Barreta et al. reported 28.6% of patients with proliferative diabetic retinopathy after 26 years of diabetes, Klein et al. reported 51.2% after 20 years and 67% after 35 years, and Karma et al. reported 50% after 20 years[31,33,43]. In present study all patients with over 26 years of diabetes duration had proliferative diabetic retinopathy.

Among all patients included in this study, 28 (23.3%) had MA, and 14 (50%) of them were on diabetic treatment for 15 to 25 years. Other authors reported from 5–22% of patients having MA, depending on the duration of diabetes, detecting method and patients characteristics[44,45]. Many authors report that MA is rare in first 5 years of diabetes. Persistent MA was found in patients over 10 years of diabetes, with greatest incidence (27.8%) in patients with 21–25 years of diabetes treatment, which was the same as other authors previously reported[24,45].

The incidence of nephropathy was getting higher with the duration of diabetes. Clinical diabetic nephropathy was not found in patients with less than 10 years of diabetes treatment, while 9 (24.3%) had it and have had diabetes for 16–25 years. Other authors found proteinuria in 15–40% of patients with T1D, with a peak incidence around 15–20 years of diabetes duration[25,46].

In conclusion, there was a statistically significant correlation between MA and the degree of diabetic retinopathy, where higher MA is mostly accompanied by higher grade diabetic retinopathy. Proteinuria followed the same trend. The highest levels of albumine in 24 hour urine were found in patients with proliferative diabetic retinopathy. Therefore, the detection of MA, as an indicator of developing clinically evident diabetic nephropathy in patients with T1D, is the most adequate way of discovering early pathological renal changes in patients with high risk of development of diabetic nephropathy. Generally, the investigated polymorphysms in the four candidate genes did not show strong effects on the development of late complications of T1D and large sample sizes will be required to detect more subtle effects.

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POVEZANOST RETINOPATIJE I NEFROPATIJE U PACIJENATA SA DIJABETESOM MELITUSOM TIP 1 SA GENSKIM POLIMORFIZMIMA RECEPTORA ZA VITAMIN D, TNF, NEURO-D I IL-1 RECEPTOR

S A Ž E T A K

Retinopatija i nefropatija su česte kasne komplikacije dijabetesa tip 1 (engl. Type 1 diabetes -T1D). U ovoj smo studiji ispitali da li pojedinačne razlike četiriju gena kandidata značajno utječu na razvoj i progresiju kasnih komplikacija T1D. U ovu smo studiju uključili 121 pacijenta kojima je bila tražena prisutnost dijabetičke retinopatije i nefropatije. Kod 47 pacijenata ispitali smo prisutnost genotipskih varijanti za receptor vitamina D (engl. Vitamin D receptor-VDR) i faktor tumorske nekroze (Tumor necrosis factor-TNF), a gene za Neuro D1 i receptor 1 interleukina-1 (engl. Interleukin-1 receptor 1-IL1R1) u 35 pacijenata. Dijabetičku retinopatiju su imali 66 (55% pacijenata nakon medijana od 13.0 godina nakon dijagnoze. Dijabetičku nefropatiju je imalo 14 (11.66%) pacijenata, a oni su svi već imali prethodno razvijenu retinopatiju. Nađena je značajna korelacija između stupnja dijabetičke retionpatije i srednje mikroalbuminurije (MA) (χ^2=54,18, p<0,001). Nakon korekcije u odnosu na duljinu bolesti nađeno je da samo gen za VDR zvan Bsml, ima značaju korelaciju sa kumulativnom prevalencijom dijabetičke retinopatije, dok niti jedan ispitani genski polimorfizam nije mogao sa sigurnošću predvidjeti nastanak dijabetičke retinopatije.