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GENETICS

The rs3917779 polymorphism of P-selectin's significant association with proliferative diabetic retinopathy in Yazd, Iran

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Abstract
Purpose This study aims at investigating possible associations of P-selectin polymorphisms with proliferative diabetic retinopathy (PDR) in Yazd, Iran.
Methods The subjects of the study included of 55 PDR and 55 diabetic no retinopathy (DNR) cases attending Yazd Diabetes Research Center (YDRC). P-selectin genotyping was done by an ARMS-PCR method.
Results The P-selectin variants rs6128, rs6133, and rs3917779 were not in Hardy-Weinberg equilibrium. The frequency of the rs3917779 C allele ($P<0.0001$), but not the rs6133 G allele ($P=0.19$) or rs6128 allele ($P=0.20$), was higher in PDR cases than in control DNR cases. Significant differences in the distribution of rs3917779 ($P<0.001$), but not rs6128 ($P=0.52$) or rs6133 ($P=0.18$), genotypes were observed between cases and controls, and only rs3917779 showed a significant association with PDR, with increments of 49.2 (14.72–125.07) in disease risk seen for CC genotypes. Among the eight three-locus P-selectin haplotypes constructed (rs6128 / rs6133 / rs3917779), there was no significant difference between frequencies of haplotypes in the DNR and PDR groups.
Conclusions P-selectin gene polymorphisms and haplotypes can contribute to PDR development.

Keywords P-selectin · Polymorphism · rs3917779 · Proliferative diabetic retinopathy

Introduction

Diabetes mellitus type II affects many people worldwide and diabetic retinopathy (DR), the major cause of blindness amongst middle-aged people, is one of its most important clinical complications [1]. DR is a kind of retinal damage and usually occurs about 5 years after the onset of diabetes. DR progresses from mild to moderate and then to severe non-proliferative diabetic retinopathy (NPDR), and eventually to proliferative diabetic retinopathy (PDR). This progression is a crucial complication in 3 % of patients with diabetes approximately 11 or more years after diagnosis [2, 3]. An increase in vascular permeability causes NPDR, whereas PDR is the resultant of fibrosis and neoangiogenesis. Furthermore, the extensive proliferation of neoangiogenesis in the PDR form often leads to vitreous haemorrhage, retinal detachment, and, finally, neovascular glaucoma [4].

Environmental and genetic factors both affect the development and progression of DR into PDR. However, the understanding of genetic risk factors is still in its infancy and there is growing evidence that there exists a genetic predisposition for PDR [4]. The causal relationship between inflammation and angiogenesis in PDR is now widely accepted [5] and several gene polymorphisms have been reported to be associated with PDR and as potential genetic markers for PDR in subjects with type 2 diabetes (T2D) [4]. One group of these genes is

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the cell adhesion molecule (CAM) group. The substantial over-expression of this group has been demonstrated in fibro-vascular membranes in patients with PDR [6] and possible associations between risk genotypes of CAMs and PDR in T2D have been tested in a few studies [7, 8].

Most of the CAMs belong to four protein families including: the immunoglobulin superfamily, the integrins, the cadherins, and the selectins [9]. The selectins is a family of heterophilic CAMs that bind fucosylated carbohydrates and has three family members: E-selectin (SELE) on endothelial cells, L-selectin (SELL) on leukocytes and P-selectin (SELP) on platelets and endothelial cells [10]. SELP is the endothelial cellular adhesion molecule [10] and the expression of it on adherent platelets, and on endothelium, has been shown to contribute to leukocyte recruitment (including neutrophils, monocytes, T cells, eosinophils, basophils, platelets and some malignant cells [11]) and support their attachments [12, 13] in the site of injury during inflammation [14, 15].

Recently, a CARe study on 2691 T2D cases reported that 3 single nucleotide polymorphisms (SNPs) in P-selectin (SELP), including rs6128 (2346G>A), rs6133 (1918G>T) and rs3917779 (33530C>T), were significantly associated with DR. In this study, the SNPs of 2000 genes, including 39 genes previously reported as related genes to DR, diabetic nephropathy (DN), and T2D, were surveyed [16]. The strong and significant relationship between these polymorphisms and DR among such a big American-European population of patients with T2D, and the high prevalence of diabetes type 2 in our region (Yazd, Iran), acted as an impulse for this study to explore whether PDR complications in our ethnic patients with T2D in Yazd province shows the same association with P-selectin polymorphisms or not. To the best of our knowledge, this is the first study that investigates the association between these polymorphisms and PDR complication of T2D disease.

Methods and materials

Sample collection

A total of 110 patients with T2D (male: 62, female: 48) were enrolled for this project. Diagnosis of T2D was based on 1985 World Health Organization criteria. These patients were divided into two groups according to duration of diabetes and status of DR; the cases group had a longer duration of diabetes (>20 years) with no retinopathy (n=55), diabetic no retinopathy (DNR), while the control group had a shorter duration (<15 years) with PDR (n=55). These patients were identified through the Diabetes Research Center at Shahid Sadoughi University of Medical Sciences (Yazd, Iran) and the protocol was reviewed and accepted by the ethics committee. The cases, duration of diabetes, age, systolic and diastolic pressure as well as the level of cholesterol and triglyceride (TG) were also determined for further comparison.

DNA extraction

Genomic DNA was extracted from whole blood using an AccuPrep ® genomic DNA extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer's instruction. The purity and quality of DNA samples were also measured using a Thermo Scientific™ NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Primer design and ARMs PCR

The amplification refractory mutation system polymerase chain reaction (ARMS-PCR) method was used for genotyping of rs6128, rs6133 and rs3917779. In total, 13 primers (4 primers: rs6128; 5 primers: rs6133; 4 primers: rs3917779) were designed by PRIMER1 (http://primer1.soton.ac.uk/primer1.html) and gunrunner software (http://www.generunner.net) (Table 1). PCR reactions were accomplished in a total volume of 25 μl, consisting of DNA (50 ng/μl): 3 μl, Pre-mix (Cinnagen, Tehran, Iran): 10 μl, inner primers, outer primers and distilled water. Each amplicon conveys one of the two different alleles of SNPs in order to validate the observation. Some of the amplified sequences, with the same outer primers, were sent for sequencing to MacroGen Inc. (Seoul, Korea).

Statistical analysis

The SPSS21.0 statistical software was used to analyze the data. The data comparison of clinical parameters between the two groups was done by an independent samples t-test, and describing the data was done by $\chi 2$-test. The analysis revealed that genotype and allele frequency, utilizing the gene counting method for direct calculation, and the distribution between genotype groups compares with $\chi 2$, and inspection for the difference is statistically significant ($P<0.05$). In order to find the frequent haplotype, the Hardy-Weinberg equilibrium and to investigate genotype association with PDR, SNPstat tools were used [17].

In-silico analyses

The web based tools, including Polymorphism Phenotyping v2 (PolyPhen-2) and Sift and Mutation-t@ster, were used to anticipate the possible impact of amino acid substitution on the function and structure of P-selectin protein.

Table 1 PCR primers and conditions

SNP	Primer sequence	Temp. (°C)	T. D. PCR	Anneal. Temp. (°C)	Amplicon size
rs6128 A/G	Forward inner primer GGTGGAGCGGTGGCTTCGACG	69.2	Yes	67	330 bp (G allele)
	Reverse inner primer GGAGCGTCCCACCCATTATCAGACCTCTT	73.3			232 bp (A allele)
	Forward outer primer CAGATTTCCACACCCACTACCAAGTCTCCC	73.5			513 bp
	Reverse outer primer GTTAGCTTGTCAGCATTCCCAGGACCTTTG	72.1			
rs6133 C/G/T	Forward inner primer1 GCATAGCATCAGTTCCTACTCCAGCGG	71.3	Yes	66	297 bp (G allele)
	Forward inner primer2 GGCATAGCATCACTTCCTACTCCAGCGT	71.6			298 bp (T allele)
	Reverse inner primer GAGTGGTGAGGGCTGGACATTGGAG	70.7			606 bp (C allele)
	Forward outer primer GGTGAGACTTCAACATACAGGCACAATGG	70.4			852 bp
	Reverse outer primer TGGGGTAGAGTCACTGGTCCAGTTTTTCA	70.4			
rs3917779 C/T	Forward inner primer GAATCTCAGGTAAGTCACTTGTGAATTGAT	66.6	Yes	64	243 bp (T allele)
	Reverse inner primer GCTGCAATCTGTGGAGTGGAAAATAG	66.3			253 bp (C allele)
	Forward outer primer TTTCCTAATGGCACATGACTTGGAG	64.2			441 bp
	Reverse outer primer TCCACACAAATGACCCTTAAGTTGG	64.2			

SNP single nucleotide polymorphism, *N* sample size, *T.D. PCR* touch down polymerase chain reaction

Results

Clinical traits analysis

The analysis reveals that the mean age, sex ratio and insulin usage among the two groups have no significant differences. However, the mean of the patients' diabetes background was significantly different between the patient groups with PDR and DNR, 9.98 (±3.96) and 24.29 (±3.86), respectively. The systolic and diastolic blood pressure and TG concentration in the PDR group were also significantly higher than in the DNR group (Table 2). In spite of these clinical trait differences between two groups, only systolic blood pressure showed significant association with rs3917779. Furthermore, the TT genotype in comparison with the CC genotype showed a significant decrease in systolic blood pressure, −11.22 (−20.52 / −1.93).

Allele, genotype and haplotype analysis

The genotypes of three polymorphisms were determined and compared between the two groups. There was a significant association between CC genotype at SNP rs3917779 and PDR, however, this relation was not observed between other genotypes of two polymorphisms (rs6128 and rs6133) and PDR in our samples. The frequency of the rs3917779 C allele ($P<0.001$; OR (95 % CI) = 42.9 (14.71–125.06)) was higher in the PDR group compared to the DNR group (Table 3). Moreover, the frequent haplotype in the DNR and PDR groups was G/G/C (rs6128/rs6133/rs3917779; Table 4).

Bioinformatics analysis

Among these three polymorphisms, only rs6133G>T could be potentially functional; however, according to the PolyPhen-2 report, this polymorphism was predicted to be benign with a score of 0.009. Also, based on SIFT and Mutation T@ster prediction, rs6133G>T could be tolerated and it could be only a polymorphism.

Discussion

Several investigations have been done and replicated on the association between the polymorphisms of *SELP* and (micro- or macro-) vascular inflammatory complications in different diseases, such as atherosclerosis [18], thrombosis [19], and myocardial infarction [20]. However, only a few studies [21] have focused on the contribution of *SELP* polymorphisms and the risk of vascular complications in T2D patients, which justified the main objective of this study.

Table 2 Clinical characteristics of patients with T2D, *t*-Test (df=108)

Clinical characteristics	Group	N	Mean	SD	SEM	Difference	95 % CI	t.t. *P*-value
Systolic pressure	PDR	55	143.73	25.04	±3.38	9.82	(1.52)–(18.12)	t=2.3454
	DNR	55	133.91	18.35	±2.47	s.e.=4.19		p=0.0208
Diastolic pressure	PDR	55	85.12	11.70	±1.58	5.19	(1.20)–(9.17)	t=2.5781
	DNR	55	80.00	9.23	±1.24	s.e.=2.01		p=0.0113
Age	PDR	55	58.80	10.91	±1.47	−2.34	(−6.02)–(1.32)	t=1.2670
	DNR	55	61.14	8.33	±1.12	s.e.=1.85		p=0.2079
Duration of diabetes	PDR	55	9.98	3.96	±0.53	−14.310	(−15.79)–(−12.83)	t=19.1958
	DNR	55	24.29	3.86	±0.52	s.e.=0.74		p<0.0001
Cholesterol	PDR	55	201.70	52.96	±7.14	5.42	(−16.30)–(27.13)	t=0.4946
	DNR	55	196.29	61.61	±8.31	s.e.=10.95		p=0.6219
Triglyceride	PDR	55	225.02	97.26	±13.11	40.22	(7.09)–(73.34)	t=2.4067
	DNR	55	184.80	76.81	±10.36	s.e.=16.71		p=0.0178

DNR diabetic no retinopathy, *PDR* proliferative diabetic retinopathy, *SD* standard deviation, *SEM* standard difference of the mean, *CI* confidence interval, *t.t.* t-test

Different clinical studies have previously established that leukocyte adhesion molecules play an important role in recruitment of leukocytes to inflammatory and hemorrhagic sites [22]. Moreover, the potential function of these molecules have also been reported during both stages of DR (NPDR and PDR). The up-regulation of *SELP*, as an important subgroup of this protein family, has also been detected in the choroidal vessels of diabetic patients [17] and especially in diabetic patients with a PDR complication [23]. Some other studies using animal models of early DR have alternatively confirmed the involvement of SELP in leukocytes, adhering to the endothelial cells in the damage location and increasing the vascular permeability of retinal vessels [24, 25]. This phenomenon has been associated with the presence of leukocytes in fibrovascular membranes, as a characteristic feature of the pathologic changes, and PDR [26]. Taken together, these lines of evidence indicate that the evoked leukocytes by SELP disrupt the homeostasis of the vasculature and facilitate proliferative damage in DR and convert it to PDR.

Nevertheless, the first study that acclaimed the presence of a significant association between *SELP* polymorphisms and DR risk in patients with T2D was Sobrin et al.'s (2011) study [16]. In her second published article in this field, she recently acclaimed the significant association between SELP plasma levels and genetic variants with DR in African Americans [27]. To the best of our knowledge, this case control study is the first to address the contribution of *SELP* polymorphisms (rs6128, rs6133 and rs3917779) to the risk of PDR in comparison with the diabetic patients without DR in an Asian population.

The genotype and allele frequencies of these three polymorphisms in this study showed deviation from the Hardy-Weinberg equation. Moreover, according to our results, the inbreeding coefficients of the study's sample population, in relation to three polymorphisms, were high (rs6128=23 %,

Table 3 P-Selectin genotype frequencies and Hardy-Weinberg equilibrium of T2D patients

SNP	Genotype	Observed	Expected	HWE (X^2 test)[a]	Inbreeding coefficient	DNR	PDR	OR (95 % CI)	*P*-value
rs6128	G/G	51	45.2	5.80 (P=0.016)	23 %	28 (0.29)	23 (0.26)	1.00 (reference)	0.52
	A/G	39	50.6			19 (0.48)	20 (0.54)	1.28 (0.56–2.96)	
	A/A	20	14.2			8 (0.23)	12 (0.20)	1.83 (0.64–5.22)	
rs6133	G/G	66	53.2	33.19 (P=0.000)	55 %	13 (0.13)	32 (0.11)	1.00 (reference)	0.18
	G/T	21	46.6			34 (0.50)	8 (0.56)	0.65 (0.24–1.79)	
	T/T	23	10.2			8 (0.37)	15 (0.33)	1.99 (0.74–5.33)	
rs3917779	CC	74	49.8	110 (P=0.000)	100 %	21 (0.38)	53 (0.96)	1.00 (reference)	<0.0001
	C/T	0	48.4			0	0	–	
	T/T	36	11.8			34 (0.62)	2 (0.04)	0.02 (0.01–0.11)	

[a] If X2 test P<0.05: not consistent with HWE.

SNP single nucleotide polymorphism, *HWE* Hardy-Weinberg equilibrium, *DNR* diabetic no retinopathy, *PDR* proliferative diabetic retinopathy, *OR* odds ratio, *CI* confidence interval

Table 4 Haplotype frequency estimation (n=110)

rs6128	rs6133	rs3917779	Total	DNR	PDR
G	G	C	0.37	0.28	0.46
G	G	T	0.14	0.28	N/A
A	G	C	0.13	0.06	0.20
A	T	C	0.10	0.03	0.17
G	T	C	0.07	0.01	0.13
A	T	T	0.07	0.11	0.03
A	G	T	0.06	0.12	N/A
G	T	T	0.06	0.11	0.01

DNR diabetic no retinopathy, *PDR* proliferative diabetic retinopathy

rs6133=55 %, and rs3917779=100 %). The consanguinity of this indigenous population (Yazd, Iran=50.2 %, coefficient of inbreeding=0.021) [28] also had a high prevalence. In spite of the high homogeneity in this ethnic population and the samples, rs3917779 showed significant differences in the distribution of genotypes between the case and control groups of the study. This difference proves the existence of a significant association between the CC genotype and an increase in systolic pressure, as much as 11.87 mmHg (P=0.0085), compared with diabetic patients without DR. Moreover, the homozygous genotype of TT also indicated a reverse association with PDR and reduction in systolic pressure. Thus, it seems that the TT genotype can be less likely to develop PDR and shows a significant association with lower systolic blood pressure.

The *SELP* gene is located in proximity of two other members of the selectin family, i. e., *SELL* and *SELE*, on chromosome 1 (1q24.2) and the association between hypertension, atherosclerosis and myocardium infarction and the level of plasma-soluble SELP and SELE and some SNPs of them have been previously acknowledged in different studies [29–32]. However, rs3917779 has not yet been included among them and the bioinformatic prediction results of this study could convey that this SNP does not have the potential to have any pathogenic effect on the splicing procedure of *SELP* mRNA. In spite of that, rs3917779 (intron 10 of *SELP* on chromosome 1), referring to ENSEMBL annotation (ENSR00000201719), is located in a CCCTC-binding factor (CTCF) binding site. CTCF is a transcriptional regulator that could be involved in different ways of transcription regulation, including selective promoter activation/repression, enhancer blocking and/or barrier insulation, hormone-responsive silencing, genomic imprinting, and alternative splicing [33, 34]. Therefore, it seems possible that the TT genotype of rs3917779 causes reduction in the *SELP* and/or other proximal selectin genes' (e.g., *SELL* and *SELE*) transcription by its impact on CTCF binding specificity.

According to another recently published study by Sobrin et al. on PDR, chromosome 1 had the highest genome-wide locus association with this complication among T2D patients; but this association was not sufficiently significant and they mentioned that "the sample size, while the largest to date, may still not be sufficient to detect an admixture mapping signal" [35]. This locus association, in spite of its low level of significance, could potentially explain the variations in different SNP association studies of the selectin gene family in association with DR progression.

Future studies can further evaluate the effects of this SNP on the regulation of selectin gene loci and the frequencies of alleles and genotypes of rs3917779 among people without diabetes. Besides, it can prove to be necessary for interested researchers to further explore a larger and more generalizable sample population for genotyping in order to elevate the reliability and reputability of the results of their analyses.

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Conflict of interest All authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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