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ORIGINAL ARTICLE

Increase levels of apo-A1 and apo B are associated in knee osteoarthritis: lack of association with *VEGF*-460 T/C and +405 C/G polymorphisms

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Abstract To investigate the genotype and allele frequency of vascular endothelial growth factor gene polymorphisms in knee osteoarthritis (OA) and their relationship with disease activity and lipid profile, we enrolled 49 knee OA patients and 75 healthy subjects (HS) as a control group. Body mass index (BMI), laboratorial assessment and genotyped by polymerase chain reaction–restriction fragment length polymorphisms (PCR–RFLP) were studied in both groups. Disease activity was determined using Lequesne and WOMAC indexes; a P value < 0.05 was considered significant. The −460 and +405 *VEGF* polymorphisms did not shown significant association between OA patients and HS. However, between OA patients and HS a significant differences were observed in BMI, age, apo A-I and apo B, independently of both polymorphisms studied ($P < 0.05$). In conclusion, increased apo A-1 and apo B levels are associated in knee OA, but the −460 T/C and +405 C/G *VEGF* polymorphisms are not associated with knee OA susceptibility.

Keywords Vascular endothelial growth factor · Polymorphism · Osteoarthritis

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Introduction

Osteoarthritis (OA) is a chronic, painful, disabling disease that affects synovial joints, is the most common joint disorder of people over the 65-years-old [1–3]. Cartilage degradation, synovial inflammation, angiogenesis and osteophyte formation are some of the key characteristics in OA [3–6]. Although considerable knowledge is available about pathology of OA, underlying molecular mechanisms regulating this disease are still not clear.

Vascular endothelial growth factor (VEGF) is a disulphide linked homodimer of 34–48 kD, glycoprotein of two 121–206 residues subunits generated by alternative splicing from a single *VEGF* gene [6, 7, 9]. This cytokine is the major proangiogenic factor involved in angiogenesis in many tissues, including cartilage [8, 9].

The *VEGF* human gene is located in the chromosome 6p12–21.3; consist of eight exons and seven introns [10]. Several polymorphisms have been described including −460 T/C and +405 C/G. These polymorphisms have been associated with diabetic retinopathy, prostate cancer, spontaneous preterm delivery, giant cell arteritis, endometriosis, rheumatoid arthritis, Behcet and Kawasaki diseases [11–17]. The aim of this study was to identify the genotype and allele frequency of −460 T/C and +405 C/G polymorphisms and their relationship with disease activity and lipid profile in knee OA.

Materials and methods

Study design

This was a case-control study.

Ethical consideration

This study was conformed to the ethical guidelines of the Helsinki 2004 Declaration and was approved by the ethics committee of the Hospital Civil "Fray Antonio Alcalde". All participants provided written informed consent prior to their enrollment into the study.

Patients and healthy subjects groups

Forty-nine knee OA patients were enrolled from the Hospital Civil "Fray Antonio Alcalde", Rheumatology Service, from December 2003 to April 2005. All patients fulfilled the 1986 classification criteria for knee OA according to American College of Rheumatology. Western Ontario and McMaster Universities (WOMAC) and Lequesne disability indexes were applied to OA patients [18, 19]. The median age in the OA group was 55 and the age ranged from 31 to 86 years. The male to female ratio was 1:23. The inclusion criteria for the study were: >30 years, no overlapping disease, and being diagnosed with primary knee OA. Seventy-five healthy subjects were included as control group. The inclusion criteria for the study were: >30 years and apparently clinically healthy. All individuals were adults Mexican Mestizo residents from Guadalajara, Jalisco, Mexico.

Laboratory assessment

Serum total cholesterol (TC), triglycerides (TG), high density lipoprotein-cholesterol (HDL-c), low density lipoprotein-cholesterol (LDL-c), very low density lipoprotein-cholesterol (VLDL-c), were assayed according to SYNCHRON CLINICAL SYSTEM LX20 of FALCON methods. Apolipoprotein A-I (apoA-I), apolipoprotein B (apoB) levels were measured according to manufacturer assay (IMMAGE™ Immunochemistry, Beckman Coulter System 4700).

Genotyping of the −460 T/C and +405 C/G polymorphisms within the *VEGF* gene

Genomic DNA (gDNA) was extracted from peripheral blood samples, according to the Miller method [20]. Polymerase chain reaction (PCR) for −460 T/C in the promoter of *VEGF* gene polymorphism was carried out using the following primers, 5′ TGTGCGTGTGGGGTTGAGCG-3′ (forward) and 5′ TACGTGCGGACAGGGCCTGA 3′ (reverse) [12] in a final volume of 25 μL, containing 500 ng of gDNA, 20 μM of each primer, 1.5 U/μL *Taq* DNA polymerase (Invitrogen™life technologies), 2.5 μL of supplied 10X buffer enzyme, 1.5 mM $MgCl_2$, 2.5 mM of each dNTP (Invitrogen™ life technologies) and 5 μL of Betaine (SIGMA). PCR amplification was performed in a programmable thermal cycler Techne TC-312. The cycling conditions were set as follows: initial denaturation at 94°C for 3 min, followed by 35 amplification cycles at: 94°C during 30 s for denaturation, 60°C during 30 s for annealing and 72°C during 30 s for extension, and finally, 72°C during 1 min for ending extension. The PCR product resulted in a 175-bp amplified fragment analyzed on a 2% agarose (Invitrogen™ life technologies) gel stained with ethidium bromide. The amplified fragment was incubated with 3 U of *BstU I* restriction enzyme (New England BioLabs) for 30 min in a heat bath at 60°C. Restriction fragments were analyzed on a 2% agarose gel (Invitrogen™ life technologies) stained with ethidium bromide. The wild-type genotype (T/T) corresponds to 155 and 20 bp fragments; heterozygote genotype (T/C) is represent by 175, 155 and 20 bp fragments; and homozygote genotype (C/C) corresponds to 175 bp fragment. Each genotype was made by duplicate to confirm the results.

PCR for +405 C/G *VEGF* gene polymorphism was carried out using the following primers, 5′ TTGCTTGCCATT CCCCACTTGA-3′ (forward) and 5′ CCGAAGCGAGAA CAGCCCAGAA 3′ (reverse) [11] in a final volume of 25 μL, containing 500 ng of gDNA, 20 μM of each primer, 1.5 U/μL *Taq* DNA polymerase (Invitrogen™ life technologies), 2.5 μL of supplied 10X buffer enzyme, 1.5 mM $MgCl_2$, 2.5 mM of each dNTP (Invitrogen™ life technologies) and 5 μL of Betaine (SIGMA). PCR amplification was performed in a programmable thermal cycler Techne TC-312. The cycling conditions were set as follows: initial denaturation at 94°C for 3 min, followed by 35 amplification cycles at: 94°C during 30 s for denaturation, 67°C during 30 s for annealing and 72°C during 2 min for extension, and finally, 72°C during 1 min for ending extension. The PCR product resulted in a 469-bp amplified fragment analyzed on a 2% agarose (Invitrogen™ life technologies) gel stained with ethidium bromide. The amplified fragment was incubated with 3 U of *BsmF I* restriction enzyme (New England BioLabs) for 2 h in a heat bath at 65°C. Restriction fragments were analyzed on a 2% agarose gel (Invitrogen™ life technologies) stained with ethidium bromide. The wild-type genotype (C/C) corresponds to 273 and 196 bp fragments; heterozygote genotype (C/G) is represent by 469, 273 and 196 bp fragments; and homozygote genotype (G/G) corresponds to 469 bp fragment. Each genotype was made by duplicate to confirm the results.

Statistical analysis

Genotype and allele frequencies differences between groups were tested using Chi-square test (χ^2), odds ratio (OR) with 95% confidence interval (MedCalc®Statistical Software). A student *t* test was used for two-group means comparison using SPSS 10.0 software and ANOVA-one way were used to compare the laboratorial assessment according each genotype. Probability values <0.05 were considered significant.

Results

Clinical and demographic characteristics

The clinical and demographic characteristics in both studied groups are shown in the Table 1. However, only the BMI and the age were significantly higher in OA than HS ($P = 0.001$). Respect to lipid profile apo A-I and apo B were significantly increased in OA patients versus HS ($P < 0.05$; Table 2). The other biochemical parameters not showed significant differences.

Genotype and allele frequencies of *VEGF* −460 and +405 polymorphisms

The genotype and allele frequencies for the −460 and +405 polymorphisms are presented in the Table 3. The genotype frequencies were in agreement with Hardy–Weinberg equilibrium ($P > 0.05$). In relationship to −460 polymorphism, the heterozygous T/C genotype was the most frequent in the both groups and the homozygous T/T genotype was present in 41% in OA and HS, without significant differences. The homozygous C/C polymorphic genotype was present in 8% (OA) and 16% (HS), respectively.

When we analyzed the relationship between −460 T/C polymorphism with the lipid profile in OA versus HS, a significant difference between C/C −460 carriers and high levels of TG and VLDL-c ($P < 0.05$), was found in HS (Fig. 1).

On the other hand, the +405 G/G genotype in OA patients was found in 29% compared to 27% in HS. In addition, the homozygous C/C was more frequent in OA (49%, $n = 24$) versus HS (44%, $n = 33$). The genotype frequency of the heterozygous was 22% in OA patients versus 29% in HS, without significant differences.

No significant differences were found between +405 genotypes and lipid profile (data not shown). The allele frequencies in both *VEGF* polymorphisms did not show significant differences (Table 3).

Discussion

We observed an association between BMI and knee OA. These results are in accordance with the consistent evidence of high rates of knee OA in overweight persons [21–23]. There is a still ongoing debate about the contribution of biomechanical, systemic or metabolic factors to explain the high risk of OA in oversized persons. However, the mechanical effects of obesity appear to be the most plausible explanation [20].

Our OA patients showed higher apo AI and apo B levels versus HS. Apo B serves as an essential structural component of chylomicrons, VLDL, intermediate density lipoprotein (IDL) and LDL. In addition, apo B is a ligand for LDL receptor that facilitates cholesterol delivery to the tissues and promotes cholesterol accumulation in the arterial tissue being modified by oxidation and (or) specific binding to extracellular matrix proteoglycans (atherogenic effect). In contrast, apo A-I is structural component of HDL, mediates

Table 1 Demographic and clinical characteristics in OA patients and HS

	OA ($n = 49$)	HS ($n = 75$)	*P*
Demographics			
Age, years	55.37 (31–86)	45.05 (31–69)	0.001
Female/male ratio	47:2	56:19	–
Disease status			
Disease duration, years	4.81 (0.5–20)	–	–
Drug treatment			
NSAIDs	41/49	–	–
Clinical assessment			
BMI (kg/m^2)	29.9 (17.5–46.9)	27.0 (19.3–37.2)	0.003
Patient's global assessment of disease status (0–10, VAS)	5.24 (0–10)	–	–
WOMAC score	2.46 (0.83–3.94)	–	–
Lequesne score	12 (3–21)	–	–

Values are reported in mean and range

OA osteoarthritis, *HS* healthy subjects, *NSAIDs* non steroidal anti-inflammatory drugs, *BMI* body mass index, *VAS* visual analogue scale, *WOMAC* Western Ontario and McMaster Universities index

Table 2 Laboratorial assessment in OA patients and HS

LA	OA ($n = 49$)	HS ($n = 75$)	P
TC, mg/dL	205.53 (45.75)	207.36 (47.02)	NS
TG, mg/dL	156.90 (84.52)	126.32 (73.47)	NS
HDL-c, mg/dL	43.66 (13.65)	44.06 (12.59)	NS
LDL-c, mg/dL	131.87 (38.37)	135.49 (45.9)	NS
VLDL-c, mg/dL	31.38 (16.9)	29.56 (18.44)	NS
apo A-I, mg/dL	171.59 (65.55)	143.02 (31.85)	0.002
apo B, mg/dL	134.44 (54.60)	103.9 (29.57)	0.001
apo B/apo A-I ratio	0.84 (0.3)	0.76 (0.32)	NS

Values are reported in mean (±SD)

LA laboratorial assessment, *OA* osteoarthritis, *HS* healthy subjects, *NS* not significant, *TC* total cholesterol, *HDL-c* cholesterol high density lipoprotein, *LDL-c* cholesterol low density lipoprotein, *VLDL-c* cholesterol very low density lipoprotein

Table 3 Genotype and allele frequencies of −460 T/C and +405 C/G *VEGF* polymorphisms in OA and HS

−460 T/C genotype frequency	OA ($n = 49$) % (n)	HS ($n = 75$) % (n)	P value
T/T	41 (20)	41 (31)	NS
T/C	51 (25)	43 (32)	
C/C	8 (4)	16 (12)	
Allele frequency			
p (T)	66 (65)	63 (94)	NS
q (C)	34 (33)	37 (56)	
+405 C/G genotype frequency			
C/C	49 (24)	44 (33)	NS
C/G	22 (11)	29 (22)	
G/G	29 (14)	27 (20)	
Allele frequency			
p (C)	60 (59)	59 (88)	NS
q (G)	40 (39)	41 (62)	

OA osteoarthritis, *HS* healthy subjects, *NS* not significant

efflux of cholesterol from the membrane of peripheral cells. In addition, apo A-I is an activator of lecithin-cholesterol acyltransferase, a key enzyme in the reverse transport of cholesterol from the peripheral tissues to the liver (antiatherogenic effect). Thus, plasma concentrations of these two apolipoproteins and their relative proportion may reflect cholesterol transport to the peripheral tissues, including the arterial wall [24, 25]. A high proportion of apo B/apo A-I ratio was observed in OA patients, this ratio is considered to reflect prominent cholesterol transport to peripheral tissue [26]. Other possible explanation respect to the high ratio of apoB/apo A-I is related to the age, because it has been reported that these apolipoproteins increase gradually over lifetime [24].

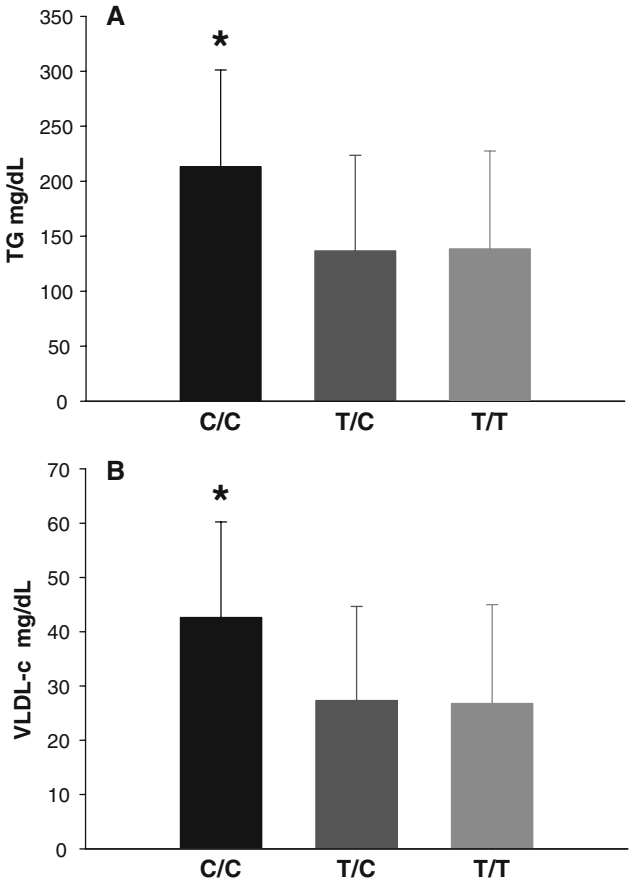

Fig. 1 −460 T/C *VEGF* polymorphism according TG and VLDL-c in HS. *$P < 0.05$ versus T/C and T/T genotypes

On the other hand, angiogenesis plays a critical role in the pathogenesis of the OA. This process is regulated by several growth factors, among which VEGF plays a central role. Approximately 70 functional polymorphisms in the *VEGF* gene have been reported [27]. The −460 T/C and +405 C/G *VEGF* polymorphisms have been associated with increased risk for many pathologies such as diabetes and its late complications [11, 27–29], breast cancer [30, 31], prostate cancer [12], lung cancer [32], sarcoidosis [33], chronic kidney disease [34], pre-eclampsia [35], rheumatoid arthritis [17], and others, however, no previous studies have reported the genotype and allele frequency of *VEGF* gene polymorphisms in OA patients.

Respect to −460 T/C *VEGF* polymorphism, similar distribution in both groups studied were found. The function of −460 T/C polymorphism remains unclear although previous evidence suggest that this single nucleotide polymorphism is associated with increased promoter activity, specially −460C allele [30, 34].

According to +405 C/G polymorphism, we identified a similar frequency in both groups studied without significant differences. However, the +405C allele and genotype G/C

have been associated with increased VEGF protein expression [36], whereas +405 G/G was linked with low VEGF protein expression in non-small cell lung cancer [36]. Moreover, in PBMC stimulated with lipopolysaccharide the +405G allele has been associated with high production of VEGF protein [34, 37]. However, internal ribosome entry site B (IRES-B) activity is increased in constructs were the promoter of VEGF +405C allele is contained [34].

On the other hand, Awata T et al. in 2002 reported that the C/C genotype is associated with high protein production in HS. This apparent inconsistency between studies may be explained in part by different designs.

These data suggest that the polymorphism changes themselves have a regulatory function or, alternatively, there is an allelic linkage between these polymorphisms and functional polymorphisms elsewhere in the gene. In conclusion, the −460 and +405 *VEGF* polymorphisms are not related with knee OA, but increased apo-Al and apoB levels are associated with knee OA.

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