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Geni renin-angiotenzinskega sistema, rastnih dejavnikov in lipooksigenazne poti ter ateroskleroza vratnih arterij pri sladkorni bolezni tipa 2

Genes of renin-angiotensin system, growth factors and - lipoxygenase pathway and atherosclerosis of carotid arteries in type 2 diabetes

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GENI RENIN-ANGIOTENZINSKEGA SISTEMA, RASTNIH DEJAVNIKOV IN LIPOOKSIGENAZNE POTI TER ATEROSKLEROZA VRATNIH ARTERIJ PRI SLADKORNI BOLEZNI TIPA 2

rezultat lastnega raziskovalnega dela. Genetska analiza je bila narejena v Genetskem laboratoriju na Inštitutu za histologijo in embriologijo Medicinske fakultete Univerze v Ljubljani

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Vsebina

Izvleček

Sladkorna bolezen tipa 2 (SB2) je kronično presnovno obolenje. Zanjo je značilna hiperglikemija, ki nastane kot posledica nezadostne proizvodnje inzulina v trebušni slinavki oziroma zmanjšane občutljivosti tkiv na delovanje inzulina.

Znano je, da sladkorna bolezen (SB) pripomore k nastanku okvar v delovanju žilnega endotelija ter k vnetju v žilnih stenah, zato se aterosklerotični procesi pri bolnikih s SB pojavijo prej in so izrazitejši.

Dosedanje raziskave so pokazale povezavo med določenimi polimorfizmi genov, katerih produkti so vključenimi v proces ateroskleroze. Aterosklerotično prizadetost vratnih arterij lahko v klinični praksi vrednotimo s kvantitativnimi meritvami posameznih aterosklerotičnih označevalcev. Najpomembnejši so debelina intime-medije (DIM), debelina plakov in seštevek plakov (angl. plaque score).

Postavili smo hipotezo, da so pri bolnikih s SB2 polimorfizmi izbranih genov renin-angiotenzinskega sistema, rastni dejavniki in lipooksigenazne poti povezani z neinvazivnimi označevalci ateroskleroze karotid (debelino intime-medije, debelino plakov in s seštevkom plakov na vratnih arterijah).

V prospektivno raziskavo smo vključili 595 bolnikov s SB2 in 200 oseb brez SB. Skupini sta bili primerljivi po spolu, starosti, indeksu telesne mase ter vrednostih sistoličnega in diastoličnega krvnega tlaka. Opravili smo meritve označevalcev ateroskleroze karotid (debelino intime-medije, debelino plakov in seštevek plakov na vratnih arterijah). Glede na ultrazvočne značilnosti smo razdelili aterosklerotične plake v pet skupin. Razširjenost ateroskleroze smo vrednotili s seštevkom plakov.

Vse meritve smo opravili ob vključitvi v raziskavo in na kontrolnem pregledu čez 3,8 ± 0,5let.

S pomočjo metode polimerazne verižne reakcije (PCR) v realnem času smo analizirali polimorfizme rs 2010963 gena VEGF, rs2071559 gena KDR, rs4646994 in rs4341 gena AK, rs12762303 gena ALOX5, rs3802278 gena ALOX5AP, rs699 in rs4762 gena za AGT, rs275651, rs931490 in rs5182 gena AT1R.

Z multivariatno regresijsko analizo smo ugotovili, da ima polimorfizem rs2071559 gena za »kinase insert domain containing receptor« (KDR) manjši vpliv na označevalce karotidne ateroskleroze (debelina intime-medije, seštevek debeline plakov), nismo pa uspeli dokazati, ali polimorfizem rs2010963 gena za žilni endotelijski rastni dejavnik (VEGF) vpliva na označevalce karotidne ateroskleroze pri bolnikih s SB2.

Dokazali smo, da ateroskleroza hitreje napreduje pri bolnikih s SB2 z genotipom DD polimorfizma rs4646994 gena za angiotenzinsko konvertazo (AK). Nismo pa uspeli dokazati, ali pri bolnikih s SB2 polimorfizmi rs4646994 in rs4341 gena AK vplivajo na debelino intime-medije karotidnih arterij.

Uspešno smo dokazali obstoj povezave med polimorfizmom rs699 gena za angiotenzinogen (AGT) in debelino plakov v karotidnih arterijah, ni pa nam uspelo dokazati obstoja povezave med polimorfizmom rs4762 gena za AGT in označevalci karotidne ateroskleroze pri bolnikih s SB2.

Dokazali smo obstoj povezave med polimorfizmoma rs275651 in rs931490 gena za angiotenzin II tip 1 receptor (AT1R) s porastom debeline plakov v karotidnih arterijah pri bolnikih s SB2.

Dokazali smo obstoj povezave med polimorfizmom rs3802278 gena za arahidonski 5-lipooksigenazni aktivacijski protein (ALOX5AP) in debelino intime-medije v karotidnih arterijah, ni pa nam uspelo dokazati obstoja povezave med polimorfizmom rs12762303 gena za arahidonsko 5-lipooksigenazo (ALOX5) in aterosklerozo karotidnih arterij pri bolnikih s SB2.

Zaključimo lahko, da polimorfizmi rs2071559 gena KDR, rs4646994 gena AK, rs699 gena AGT, rs275651 in rs931490 gena AT1R, rs3802278 gena ALOX5AP vplivajo na posamezne označevalce ateroskleroze (DIM, debelina plakov, seštevek plakov), ki smo jih v naši raziskavi kvantitativno vrednotili pri bolnikih s SB2.

Abstract

Type 2 diabetes represents a chronic metabolic disorder. It is characterized by hyperglicemia, which is a consequence of insufficient insulin production in the pancreas or of reduced tissue sensitivity to insulin actions.

It is well known that type 2 diabetes impaires the functioning of the vascular endothelium and causes inflammation in the vessel wall leading to the atherosclerotic process. For these reasons, atherosclerotic complications occur much earlier in diabetic patients than in people without diabetes.

Previous studies have shown a connection between certain polymorphisms of genes whose products are involved in the process of atherosclerosis. Atherosclerosis of carotid arteries can be evaluated in clinical practice with quantitative measurement of atherosclerotic markers. The most important ones are intima media thickness (IMT), plaque thickness and the sum of plaques (plaque score).

Our hypotheses were that in patients with type 2 diabetes (T2DM) polymorphisms of genes of renin-angiotensin system, growth factors and lipoxygenase pathway are related to the subclinical markers of carotid atherosclerosis, such as intima media thickness, plaque thickness and plaque score.

The prospective study included 595 patients with T2DM, with 200 people without diabetes representing the control group for the estimation of Hardy-Weinberg equilibrium and for the comparison of subclinical markers of carotid atheorsclerosis. We performed measurements of IMT in both left and right carotid arteries and color-coded double Doppler ultrasound examination. We divided the atherosclerotic plaques into 5 groups according to the ultrasonic characteristics. The prevalence of atherosclerosis was evaluated by the plaque score.

Blood samples were submitted to genetic analysis of certain polymorphisms by PCR and laboratory examination.

All measurements were performed at enrolment in the study and at re-examination after 3.8 ± 0.5 years.

Using the PCR method, we analyzed the rs 2010963 polymorphisms of the gene VEGF, rs2071559 KDR gene, rs4646994 and rs4341 AK gene, rs12762303 gene ALOX5, rs3802278 gene ALOX5AP, rs699 and rs4762 AGT gene, rs275651, rs931490 and rs5182 AT1R gene.

Our multivariate regression analysis showed a minor impact of polymorphism rs2071559 gene "kinase insert domain containing receptor" (KDR) on markers of carotid atherosclerosis (IMT, plaque score), but we were not able to demonstrate any impact of polymorphism rs2010963 gene VEGF on markers of carotid atherosclerosis in patients with T2DM.

We showed faster progression of atherosclerosis in patients with T2DM with DD genotype rs4646994 polymorphism of the gene angiotensin converting encime (ACE), but we were not able to demonstrate any impact of polymorphisms rs4646994 and rs4341 ACE gene on IMT of the carotid arteries in patients with T2DM.

We managed to establish a link between the rs699 polymorphism of the gene for angiotensinogen (AGT) and plaque thickness in the carotid arteries, but we found no link between the rs4762 polymorphism of AGT gene and markers of carotid atherosclerosis in patients with T2DM.

We showed a link between polymorphisms rs275651 and rs931490 gene for the angiotensin II type 1 receptor (AT1R), with an increase in plaque thickness in the carotid arteries in patients with T2DM.

We proved a link between the polymorphism rs3802278 gene arachidonic 5-lipoxygenase activation protein (ALOX5AP) and the IMT in the carotid arteries, but we did not manage to prove a link between the polymorphism rs12762303 gene arachidonic 5-lipooksigenase (ALOX5) and atherosclerosis of the carotid arteries in patients with T2DM.

We can conclude that polymorphisms rs2071559 gene KDR, rs4646994 gene ACE rs699 gene AGT, rs275651 and rs931490 gene AT1R, rs3802278 gene ALOX5AP have an impact on the individual markers of atherosclerosis (IMT thickness of plaques, the sum of plaques), which were quantitatively evaluated in our study.

Uvod

Ateroskleroza je kronična vnetna bolezen, ki predstavlja vodilni vzrok obolevnosti in umrljivosti v razvitem svetu. Bolniki s sladkorno boleznijo (SB) imajo veliko večje tveganje za nastanek ateroskleroze in z njo povezanih zapletov v primerjavi z osebami brez SB (Goldstein in sod, 2011).

Ključno patološko dogajanje pri aterosklerozi je poškodba endotelija arterijske stene oziroma vnetni odgovor, ki ga sproži poškodba. Možni povzročitelji poškodbe endotelija so SB, hiperholesterolemija, s kajenjem povezani prosti radikali, arterijska hipertenzija, različni genetski dejavniki, okužbe oziroma prepletanje omenjenih in drugih dejavnikov tveganja (Ross, 1999).

SB je kronična presnovna motnja, za katero je značilna povišana raven glukoze v krvi (hiperglikemija). Slednja je lahko posledica nezadostnega izločanja inzulina, njegovega neučinkovitega delovanja ali obojega. V grobem delimo SB na tip 1 in tip 2 (Mrevlje, 2011). Po zadnjih podatkih je bilo leta 2013 na svetu 382 milijonov ljudi s SB in 316 milijonov ljudi z moteno glukozno toleranco (IDF, 2013). SB2 je pogostejša, povezana je z zmanjšano občutljivostjo na inzulin in se izrazi ob nezadostni kompenzatorni hiperinzulinemiji (Mrevlje, 2011).

Ateroskleroza koronarnih, cerebralnih in perifernih arterij je odgovorna za 80 % umrljivosti in 75 % hospitalizacij pri bolnikih s SB (Grundy in sod., 1999). Bolniki s SB2 imajo dva- do štirikrat večje tveganje za koronarno bolezen, poleg tega je slabša tudi prognoza akutnega miokardnega infarkta in nestabilne angine pektoris (Mrevlje, 2011). SB tudi za štirikrat poveča tveganje za možgansko kap, njena prognoza pa je slabša pri bolnikih s SB. Kljub znatnemu zmanjšanju srčno-žilne umrljivosti, ki smo mu v zadnjih desetletjih priča v razvitem svetu, ostaja umrljivost v podskupini bolnikov s SB nespremenjena. Ravno nasprotno pa se je pri bolnicah s SB umrljivost zaradi srčno-žilnih bolezni celo povečala (Gu in sod., 1999).

SB je stanje hiperglikemije, ki aktivira metabolne poti, v katerih nastajajo reaktivne kisikove spojine. Poleg hiperglikemije pri bolnikih s SB prihaja do številnih presnovnih nepravilnosti, kot sta dislipidemija in inzulinska odpornost, ki povzročata žilno disfunkcijo, in s tem večjo nagnjenost k aterosklerozi (Kannel in McGee, 1979). Žilne bolezni so vzrok za umrljivost in obolevnost večine bolnikov (Mrevlje, 2011). Kronične zaplete SB delimo na mikrovaskularne (diabetična retinopatija, nefropatija, nevropatija) ter makrovaskularne (koronarne bolezni, možganske kapi in periferne arterijske žilne bolezni). Poleg povišane koncentracije holesterola LDL, znižane koncentracije holesterola HDL, arterijske hipertenzije in kajenja je kronična hiperglikemija, ki se izraža s povečanim deležem HbA1c, neodvisni napovedni dejavnik koronarne bolezni in možganske kapi. Raziskava DCCT (The Diabetes Control and Complications Trial) je pokazala, da z dobro kontrolo krvnega sladkorja učinkovito zmanjšamo kronične zaplete SB, vendar pri bolnikih s SB tudi normalne vrednosti krvnega sladkorja ne preprečijo številnih zapletov, kar zahteva iskanje novih načinov zdravljenja. Raziskave kažejo, da ima oksidativni stres, ki generira kisikove proste radikale in ki ga povzroča hiperglikemija, ključno vlogo pri razvoju in zapletih žilnih sprememb pri bolnikih s SB (Bonetti in sod., 2003).

Endotelij predstavlja stičišče med žilno steno in krvjo ter omogoča prepoznavanje in prenos signalov iz krvi v žilno steno. Zdrav endotelij uravnava žilno homeostazo z vazodilatacijo, preprečevanjem adhezije ter migracije levkocitov in trombocitov, preprečevanjem migracije in proliferacije gladkih mišičnih celic ter s pomočjo antikoagulacijskega in fibrinolitičnega delovanja (Bonetti in sod., 2003). Endotelijske celice sintetizirajo in izločajo različne biološko aktivne snovi, ki uravnavajo angiogenezo, vnetni odgovor, hemostazo ter tonus in prepustnost žilne stene (Feletou in Vanhoutte, 2006).

Številne epidemiološke in klinične raziskave so debelino intime in medije (DIM) vratnih arterij obravnavale kot zgodnji pokazatelj sistemske subklinične ateroskleroze. Prav tako povečano DIM povezujejo z nastankom srčne kapi in ishemične možganske kapi. Z ultrazvokom ne moremo ločeno ocenjevati intime in medije, zato ju ocenjujemo skupaj. Bolniki s SB imajo pogosteje prisotno in naprednejšo obliko ateroskleroze vratnih arterij v primerjavi s osebami brez SB (Frost in sod., 2000; Lee in sod., 2007; Lacroix in sod., 2006). Zadebelitev intime in medije skupne vratne arterije je povezana z možgansko kapjo (CVI), medtem ko je zadebeljena DIM v predelu bulbusa in prisotnost leh povezana s srčno-žilnimi zapleti in ishemično srčno boleznijo (Ebrahim in sod., 1999). Normalne vrednosti DIM skupne vratne arterije pri zdravi populaciji moških in žensk starih med 17 in 65 let pri moških merijo od 0,39 do 0,70 mm, pri ženskah pa od 0,30 do 0,64 mm (Gariepy in sod., 2009). Patofiziološki proces zadebelitve DIM je drugačen od patofiziološkega procesa razvoja aterosklerotičnih leh (Johnsen in sod., 2007). Aterosklerotična leha je definirana kot fokalna zadebelitev intime in medije in meri več kot 1,2 mm (Osting in sod., 2007). Več raziskav kaže, da je prisotnost aterosklerotične lehe močnejši napovednik žilnega zapleta kot DIM. Naprednejše stopnje karotidne stenoze so pomemben dejavnik tveganja za CVI. Toda le majhen delež bolnikov z asimptomatsko karotidno stenozo utrpi CVI ali tranzitorno ishemično atako (TIA), tako da še vedno ne znamo napovedati vseh dejavnikov, ki vodijo do možganske kapi. Najverjetneje so v nastanku CVI pomembne tudi druge značilnosti plakov, kot so ulceracije, površinske nepravilnosti in morfološka sestava ter velikost plaka. Najverjetneje je potek ateroskleroze oziroma njenih zapletov s trombozo plaka podoben v karotidnih arterijah in koronarnih arterijah. V koronarnih arterijah je stabilna angina pektoris posledica stabilnih plakov, ki imajo gladko fibrozno površino brez trombusov, medtem ko nestabilno angino pektoris in miokardni infarkt povzročajo iregularni plaki s fisuro ali rupturo ter z lokalnim nastankom tromba. Na podlagi emboličnega materiala, ki so ga opazili v retinalni cirkulaciji pri bolnikih s TIA, in visoke pogostnosti cerebralnih mikroembolizmov distalno od karotidne stenoze med možgansko kapjo so sklepali, da na CVI vplivajo embolizmi. Prav tako so opazovali zmanjšanje mikroembolizmov po preboleli CVI. Z namenom, da preučimo nestabilnost plakov, bomo karotidne plake ultrazvočno ločili glede na ehogenost na pet tipov (Poucerlot in sod., 1999). Plaki tipa I in tipa II so nestabilni in predstavljajo veliko tveganje za TIA in CVI. Nekateri poročajo o trikrat večjem tveganju za možgansko kap pri nestabilnih plakih (Vouillarmet in sod., 2016). Aterosklerotski plaki pri bolnikih s SB so v večjem deležu nestabilni kot pri bolnikih brez SB (Death in sod., 2003).

Geni renin-angiotenzinskega sistema

Komponente renin-angiotenzinskega sistema in kalikrein-kininskega sistema imajo številne učinke na arterije (Rizzoni in sod., 2009; Brillante in sod., 2009). Angiotenzin II povzroča vazokonstrikcijo, povzroča okvare endotelija, povečuje prepustnost holesterola LDL skozi endotelij, spodbuja razmnoževanje gladkih mišičnih celic v arterijah in remodeliranje koronarnih arterij, aktivira adhezijske molekule, monocite in makrofage (Rizzoni in sod., 2009; Brillante in sod., 2009). Spodbuja tudi sintezo beljakovin in veziva v gladkih mišičnih celicah žil ter tako spodbuja količino zunajceličnega matriksa v žilni steni (Black et al, 1995). Angiotenzin II vpliva tudi na sistem fibrinolize, spodbuja sintezo in izločanje PAI-I v endotelijskih celicah, ki je zaviralec tPA, in tako zavira fibrinolizo (Ridker et al., 1993). V renin-angiotenzinskem sistemu ima ključno vlogo angiotenzinogen, ki ga encim renin pretvori v angiotenzin I, slednjega pa encim angiotenzin konvertaza (AK) pretvori v aktivno učinkovino angiotenzin II, ki deluje na srčno-žilni sistem prek receptorja tip 1 angiotenzina II. Genetski dejavniki vplivajo na koncentracijo angiotenzinogena (genski polimorfizem angiotenzinogena), na aktivnost AK (insercija/ delecija polimorfizem AK) in na izločanje aldosterona (Pall in sod., 2004; Jeunemaitre et al., 1997). Aldosteron ima vrsto učinkov: uravnava presnovo natrija, vpliva na znotrajcelične lipide in krvni tlak. Genetska raznolikost v uravnavanju sinteze aldosterona na ta način posredno vpliva na pojav in rast plakov ter razmnoževanje gladkih mišičnih celic žil (Pall in sod., 2004).

Geni rastnih dejavnikov

V predhodnih raziskavah je bilo ugotovljeno, da geni rastnih dejavnikov močno vplivajo na nastanek srčnega infarkta (Petrovič in sod., 2007; Kariž in sod., 2009; Petrovič, 2010). Za nekatere polimorfizme rastnih dejavnikov so ugotovili, da so funkcionalni ter da so povezani s serumskim nivojem VEGF (Petrovič in sod., 2007). Funkcionalni polimorfizmi so naslednji: polimorfizma 634 C/G in +936 C/T gena za žilni endotelijski rastni dejavnik, polimorfizem insercija/delecija gena za žilni endotelijski rastni dejavnik ter polimorfizmi -834T/A, -553T/A in -921C/G gena za beta fibroblasti rastni dejavnik

Geni lipooksigenazne poti

Klinične raziskave kažejo, da na potek ateroskleroze vpliva lipooksigenazna pot (Jala in sod., 2004). Na tej poti so prisotni številni proteini, kot je npr. FLAP (aktivirajoči protein 5-lipooksigenaze) (Jala in sod., 2004; Shah in sod., 2008). Protein FLAP kodira gen ALOX5AP, ključno vlogo pri sintezi levkotrienov pa imata ALOX5AP in ALOX5 (Jin in sod., 2010). Družinske študije (GENECARD, 1101 družin) so pokazale, da je gen ALOX5AP povezan s pojavom zgodnje koronarne bolezni (Shah in sod., 2008). V raziskavah so prav tako pokazali, da je genska variabilnost v tem genu povezana z restenozo po stentiranju koronark, s koronarno boleznijo pri kavkaški rasi ter z vulnerabilnimi plaki na karotidah pri Kitajcih (Shah in sod., 2008; Crosslin in sod., 2009; Jin in sod., 2010). Prav tako je bilo ugotovljeno, da sta polimorfizma gena za ALOX5 in 5- lipooksigenaze (rs12762303, rs2660899) vpletena v patogenezo srčnega infarkta (Zsai in sod., 2007; Assimes in sod., 2008; Maznycka in sod., 2008).

Hipoteze doktorske disertacije

- Polimorfizmi testiranih genov renin-angiotenzinskega sistema, rastnih dejavnikov in lipooksigenazne poti so povezani z DIM, debelino plakov in s seštevkom plakov (angl. plaque score) na vratnih arterijah ter z bolj nestabilnimi plaki pri bolnikih s SB2.
- Genetska raznolikost testiranih genov renin-angiotenzinskega sistema, rastnih dejavnikov in lipooksigenazne poti vpliva na napredovanje aterosklerotičnega procesa pri bolnikih s SB2, ki ga bomo opredeliti z DIM, debelino plakov in s seštevkom plakov.

Cilji in namen doktorske disertacije

- Ugotoviti, ali so polimorfizmi testiranih genov renin-angiotenzinskega sistema, rastnih dejavnikov in lipooksigenazne poti povezani z DIM, debelino plakov in s seštevkom plakov na vratnih arterijah ter z bolj nestabilnimi plaki pri bolnikih s SB2.
- Ugotoviti ali genetska raznolikost testiranih genov renin-angiotenzinskega sistema, rastnih dejavnikov in lipooksigenazne poti vpliva na napredovanje aterosklerotičnega procesa pri bolnikih s SB2, ki ga bomo po dvoletnem opazovalnem obdobju opredelili z debelino intime-medije, debelino plakov in s seštevkom plakov.

Raziskava 1

POLIMORFIZMI GENOV ZA ŽILNI ENDOTELIJSKI RASTNI DEJAVNIK (RS2010963) IN NJEGOV RECEPTOR (RS2071559) IN MARKERJI KAROTIDNE ATEROSKLEROZE PRI BOLNIKIH S SLADKORNO BOLEZNIJO TIPA 2

VASCULAR ENDOTHELIAL GROWTH FACTOR GENE POLYMORPHISM (RS2010963) AND ITS RECEPTOR, KINASE INSERT DOMAIN-CONTAINING RECEPTOR GENE POLYMORPHISM (RS2071559), AND MARKERS OF CAROTID ATHEROSCLEROSIS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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Research Article

Vascular Endothelial Growth Factor Gene Polymorphism (rs2010963) and Its Receptor, Kinase Insert Domain-Containing Receptor Gene Polymorphism (rs2071559), and Markers of Carotid Atherosclerosis in Patients with Type 2 Diabetes Mellitus

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Background. The current study was designed to reveal possible associations between the polymorphisms of the vascular endothelial growth factor (VEGF) gene (rs2010963) and its receptor, kinase insert domain-containing receptor (KDR) gene polymorphism (rs2071559), and markers of carotid atherosclerosis in patients with type 2 diabetes mellitus (T2DM). *Patients and Methods*. 595 T2DM subjects and 200 control subjects were enrolled. The carotid intima-media thickness (CIMT) and plaque characteristics (presence and structure) were assessed ultrasonographically. Biochemical analyses were performed using standard biochemical methods. Genotyping of VEGF/KDR polymorphisms (rs2010963, rs2071559) was performed using KASPar assays. *Results*. Genotype distributions and allele frequencies of the VEGF/KDR polymorphisms (rs2010963, rs2071559) were not statistically significantly different between diabetic patients and controls. In our study, we demonstrated an association between the rs2071559 of KDR and either CIMT or the sum of plaque thickness in subjects with T2DM. We did not, however, demonstrate any association between the tested polymorphism of VEGF (rs2010963) and either CIMT, the sum of plaque thickness, the number of involved segments, hsCRP, the presence of carotid plaques, or the presence of unstable carotid plaques. *Conclusions*. In the present study, we demonstrated minor effect of the rs2071559 of KDR on markers of carotid atherosclerosis in subjects with T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is considered a major epidemic of this century. It is estimated that its prevalence will increase worldwide from 371 million people in 2013 to 552 million people in 2030 [1]. T2DM is associated with accelerated progression of atherosclerosis, the major cause of vascular complications leading to increased morbidity and mortality [2].

Chronic, low-grade inflammation has been demonstrated to be involved in the pathogenesis of atherosclerosis in subjects at high risk to develop cardiovascular disease [3–7]. Among immune cells infiltrating atherosclerotic lesions, polymorphonuclear neutrophil leukocytes with their products were reported to have an important role in the development and progression of atherosclerosis [8–11]. Marino and coworkers have recently reported that both circulating and intraplaque polymorphonuclear neutrophil leukocytes from

subjects with carotid atherosclerosis are active producers of different inflammatory mediators including the vascular endothelial growth factor (VEGF) [11].

Several environmental and genetic factors (i.e., hypoxia, hyperglycemia, oxidative stress, ischemia, and gene polymorphisms of VEGF) influence plasma VEGF levels [12–16]. Among several polymorphisms of the VEGF gene, the rs2010963 (−634C/G polymorphism of the VEGF gene) and few others were reported to affect serum VEGF levels [13–15]. Moreover, rs2010963 was demonstrated to be associated with several disorders, such as diabetic retinopathy, diabetic nephropathy, myocardial infarction, and impaired prognosis in patients with chronic heart failure [13–15, 17]. Despite these findings, however, data about VEGF polymorphisms and their possible association with carotid atherosclerosis in patients with diabetes mellitus are limited [18–20]. Additionally, CIMT is highly heritable and associated with stroke and myocardial infarction, making it a promising quantitative intermediate phenotype for genetic studies of vascular disease [21].

The present study was thus designed to investigate the association between polymorphisms of the VEGF gene (rs2010963) and the KDR gene (rs2071559) and markers of carotid atherosclerosis (such as carotid intima-media thickness (CIMT), the number of affected segments of carotid arteries, and the sum of plaques thickness) in patients with T2DM.

2. Material and Methods

The study protocol was approved by the Slovene Medical Ethics Committee in September 2010 (Protocol number 128/09/2010). After an informed consent for the participation in the study was obtained, a detailed interview was made.

This cross-sectional study included 595 subjects with T2DM and 200 subjects without T2DM (control group). They were selected among patients admitted to the diabetes outpatient clinics of the General Hospitals Murska Sobota and Slovenj Gradec, Slovenia. Subjects in the control group were not allowed to have T2DM, and they were the staff of the General Hospital Murska Sobota. Subjects with T2DM and control subjects were excluded if they had homozygous familial hypercholesterolaemia or a previous cardiovascular event such as myocardial infarction or a cerebral stroke.

All ultrasound examinations were performed by two experienced doctors blinded to the participants' diabetes status. The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured, as described previously [22]. Plaques were defined as a focal intima-media thickening and divided into 5 types according to their echogenic/echolucent characteristics, as previously described [22]. The interobserver reliability for carotid plaque characterization was found to be substantial ($\kappa = 0.64$, $p < 0.001$).

The genomic DNA was extracted from 100 μL of whole blood using a FlexiGene DNA isolation kit, in accordance with the recommended protocol (Qiagene GmbH, Hilden, Germany).

For VEGF rs2010963 polymorphism competitive allele specific PCR (KASP) was conducted on an ABI Step-One System (Applied Biosystems, Foster City, CA). The reaction mixture (5 μL) contained 2.5 μL 2x KASPar reaction Mix (v3), 0.07 μL Assay Mix, 1.43 μL of distilled water Dnase/RNase-free (Gibco, Invitrogen Life Technologies), and 10 ng of extracted genomic DNA (1 μL). Thermal cycling employed the following conditions: hot-start enzyme activation (15 min at 94°C), denaturation (20 sec at 94°C) followed by 10 cycles of touchdown over 65–57°C for 60 sec (dropping 0.8°C per cycle), and final 26 cycles (20 sec at 94°C and 60 sec at annealing temperature 57°C). For rs2071559 (KDR) everything was the same with the exception of thermal conditions. Hot-start enzyme activation (15 min at 94°C) and denaturation (20 sec at 94°C) were followed by 15 cycles of touchdown over 55–65°C for 60 sec (dropping 0.8°C per cycle) and final 26 cycles (20 sec at 93°C and 60 sec at annealing temperature 58°C).

In addition, the fasting serum VEGF levels were analyzed in 70 subjects with T2DM and in 33 subjects with T2DM. For the determination of the fasting serum VEGF concentration (isoform VEGF 165), a solid phase sandwich ELISA using two kinds of high specific antibodies (hVEGF Assay Kit, IBL Co., Ltd. Aramachi, Takasaki-shi, Gunma, Japan) was used. The respective CV (%) were between 3 and 5.5 for interassay measurements and between 2.6 and 5.3 for intra-assay measurements.

Continuous variables are expressed as means ± standard deviations. Continuous clinical data were compared using unpaired Student's t-test or analysis of variance (ANOVA). The Pearson χ^2 test was used to compare discrete variables. A two-tailed p value of less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 21 (SPSS Inc., Chicago, Ilinois, USA).

3. Results

Patients with T2DM were older, had a greater waist circumference, and had higher fasting glucose and HbA1c levels compared to controls, whereas there were no differences in BMI and systolic and diastolic blood pressure between patients with T2DM and control subjects (Table 1). Patients with T2DM had lower total, HDL, and LDL cholesterol levels and a higher triglyceride level compared to controls (Table 1). Plasma levels of inflammatory markers (i.e., hs-CRP and fibrinogen) were higher in patients with T2DM compared to controls (Table 1). Additionally, there was higher percentage of men, statin therapy, and antihypertensive therapy and lower percentage of smokers in T2DM group compared to control group (Table 1).

The genotype distributions in both patients with T2DM and controls were in Hardy-Weinberg equilibrium for both VEGF gene polymorphisms [rs2010963: T2DM (genotype frequencies: CC genotype 8.7%, CG genotype 47.1%, and GG genotype 44.2%; $\chi^2 = 3.48$; $p = 0.06$) and controls (genotype frequencies: CC genotype 9%, CG genotype 48%, and GG genotype 43%; $\chi^2 = 1.46$; $p = 0.22$)]. The genotype distributions in both patients with T2DM and controls

Table 1: Baseline characteristics of subjects with T2DM and subjects without T2DM (control group).

	Subjects with T2DM $n = 595$	Control group $n = 200$	p
Age (years)	62.39 ± 9.61	60.07 ± 9.18	0.008
Male sex (%)	338 (56.8)	92 (46.0)	0.008
Diabetes duration (years)	11.25 ± 7.88	—	—
Cigarette smoking (%)	53 (8.91)	34 (17.0)	0.002
Waist circumference (cm)	108.65 ± 12.88	93.31 ± 13.18	<0.001
BMI (kg/m^2)	31.00 ± 4.74	27.90 ± 4.42	0.16
SBP (mm Hg)	147.1 ± 19.80	143.3 ± 16.6	0.86
DBP (mm Hg)	85.78 ± 11.60	84.7 ± 11.6	0.19
Fasting glucose (mmol/L)	8.04 ± 2.57	5.27 ± 0.87	<0.001
HbA1c (%)	7.89 ± 3.56	4.79 ± 0.29	<0.001
Total cholesterol (mmol/L)	4.70 ± 1.18	5.36 ± 1.08	<0.001
HDL cholesterol (mmol/L)	1.20 ± 0.35	1.43 ± 0.37	<0.001
LDL cholesterol (mmol/L)	2.63 ± 0.94	3.24 ± 0.98	<0.001
Triglycerides (mmol/L)	1.9 (1.2–2.7)	1.3 (0.9–1.9)	<0.001
hs-CRP (mg/L)	3.5 ± 1.18	2.2 ± 1.18	<0.001
CIMT (μm)	958 ± 194	890 ± 212	0.007
Statin therapy (%)	375 (63.0)	62 (31.0)	<0.001
Antihypertensive agents (%)	499 (83.9)	58 (29%)	<0.001

Continuous variables were expressed as means ± standard deviations when normally distributed and as median (interquartile range) when asymmetrically distributed. Categorical variables were expressed as frequency (percentage). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: glycated haemoglobin; hs-CRP: high sensitivity C-reactive protein.

were in Hardy-Weinberg equilibrium for the KDR gene polymorphism [rs2071559: T2DM (genotype frequencies: CC genotype 22.0%, CT genotype 51.9%, and TT genotype 26.1%; $\chi^2 = 0.97$; $p = 0.33$) and controls (genotype frequencies: CC genotype 30.0%, CT genotype 48.0%, and TT genotype 22.0%; $\chi^2 = 0.63$; $p = 0.23$)]. No statistically significant differences in the VEGF rs2010963 and KDR rs2071559 genotype distribution frequencies were observed between T2DM patients and controls.

The observed minor allele frequency (MAF) distributions were mostly in agreement with the 1000 Genomes Project data in the European population. The C allele frequency of the VEGF rs2010963 showed no significant difference ($p = 0.79$) between patients with T2DM and controls (32.3% versus 33%). However, the C allele frequency of the KDR rs2071559 polymorphism was significantly lower ($p = 0.04$) in T2DM subjects as compared to the controls (49% versus 54%).

Higher VEGF serum levels were demonstrated in subjects with T2DM with the CC genotype (rs2010963) compared to those with other (CG + GG) genotypes (Table 2). Moreover, higher VEGF serum levels were found in subjects with the CC genotype (rs2071559) compared to those with other (CT + TT) genotypes (Table 2).

The comparison of atherosclerosis parameters was performed with regard to different genotypes of the VEGF polymorphism (rs2010963) upon enrolment. In our study, we did not demonstrate any association between the rs2010963 and either CIMT, the sum of plaque thickness, the number of involved segments, hsCRP or the presence of carotid plaques, or the presence of unstable carotid plaques (Tables 3 and 4). We did, however, demonstrate an association between the rs2071559 and either CIMT or the sum of plaque thickness in subjects with T2DM (Table 3).

4. Discussion

In our study, we demonstrated an association between the rs2071559 of KDR and CIMT in subjects with T2DM, whereas we did not demonstrate an association between tested polymorphism of VEGF (rs2010963) and CIMT. Variations in the VEGF gene were reported to be weakly associated with CIMT [19]. None of the single genotyped polymorphisms (−2578A>C rs699947, −634C>G rs2010963, and +936C>T rs3025039) were significantly associated with overall IMT in the study reported by Kangas-Kontio and coworkers [19]. The haplotype CCC, however, was associated with higher overall CIMT in women and the haplotype CCT with higher CIMT in the internal carotid artery in men [19].

Additionally, we also demonstrated an association between the rs2071559 of KDR and the sum of plaque thickness in subjects with T2DM, whereas no association between tested polymorphism of VEGF (rs2010963) and markers of carotid atherosclerosis was demonstrated. The rs2010963 polymorphism of the VEGF gene was not demonstrated to exert a significant influence on the risk of subclinical atherosclerosis manifested by the presence of endothelial dysfunction by brachial artery reactivity and increased CIMT in a series of patients with rheumatoid arthritis [23]. Contrary, the importance of VEGF and its receptor (VEGFR1) was reported by Russell and coworkers [24]. They analyzed 34 intact carotid endarterectomy specimens and compared histologically stable and unstable plaques. In unstable plaques (cap rupture/thinning) increased VEGF and receptor (VEGFR1) staining as well as increased microvessel density was demonstrated in comparison with stable carotid plaques [24]. Additionally, Marino and coworkers have recently reported that both circulating and intraplaque polymorphonuclear neutrophils (PMN) from subjects with carotid atherosclerosis are active producers of VEGF, IL-8, and elastase [11]. Moreover, an evidence is provided that these PMN have an increased ability to produce VEGF (at mRNA levels) in comparison to cells from healthy subjects. Additionally, increased VEGF mRNA occurs in both intraplaque and circulating PMN, at rest as well as after stimulation, suggesting that such functional

Table 2: VEGF serum levels in subjects with and without T2DM with regard to the rs2010963 and rs2071559 genotypes.

rs2010963	Mean (95% CI)		p	Linear trend analysis	
	CC (52)	CG + GG (543)		F	p
VEGF (ng/L)	63.5 ± 29.2	46.1 ± 22.3	**<0.01**	3.22	**0.03**
rs2071559	Mean (95% CI)		p	Linear trend analysis	
	CC (131)	CT + TT (464)		F	p
VEGF (ng/L)	69.4 ± 25.1	40.9 ± 28.3	**<0.01**	3.70	**0.02**

Table 3: Comparison of markers of carotid atherosclerosis (CIMT, sum of plaque thickness, and number of involved segments) in subjects with T2DM at the beginning of the study with regard to the rs2010963 and rs2071559 genotypes.

rs2010963	Mean (95% CI)			p	Linear trend analysis	
	CC (52)	CG (280)	GG (263)		F	p
CIMT (μm)	1045 ± 192 (969–1121)	996 ± 210 (964–1026)	1026 ± 210 (995–1058)	0.27	2.29	0.13
Sum of plaque thickness (mm)	7.58 ± 4.52 (5.67–9.49)	7.79 ± 4.28 (7.09–8.48)	8.11 ± 4.73 (7.35–8.88)	0.76	0.009	0.93
Number of involved segments	2.67 ± 1.51 (2.07–3.26)	2.48 ± 1.70 (2.24–2.73)	2.54 ± 1.60 (2.31–2.77)	0.84	0.34	0.56
rs2071559	Mean (95% CI)			p	Linear trend analysis	
	CC (131)	TC (309)	TT (155)		F	p
CIMT (μm)	1053 ± 186 (1012–1092)	1029 ± 200 (987–1070)	988 ± 219 (958–1019)	**0.04**	5.64	**0.04**
Sum of plaque thickness (mm)	8.81 ± 4.30 (7.83–9.78)	8.27 ± 4.50 (7.26–9.29)	7.31 ± 4.48 (6.61–8.00)	**0.03**	5.91	**0.02**
Number of involved segments	2.87 ± 1.41 (0.94–1.65)	2.38 ± 1.60 (0.95–1.73)	2.24 ± 1.70 (0.98–1.46)	0.64	0.22	0.83

Table 4: Comparison of markers of carotid atherosclerosis (presence of carotid plaques, presence of unstable plaques) in subjects with T2DM at the beginning of the study with regard to the rs2010963 and rs2071559 genotypes.

	rs2010963				rs2071559			
	CC (52)	CG (280)	GG (263)	p	CC (131)	TC (309)	TT (155)	p
Presence of carotid plaques n (%)	46 (88.5)	229 (81.8)	223 (84.7)		117 (89.3)	250 (80.9)	133 (85.8)	
OR (95% CI)	*	0.68 (0.46–2.57)	0.72 (0.41–1.26)	0.45	*	0.57 (0.53–2.06)	0.68 (0.34–1.34)	0.15
$p^{\dagger}$	—	0.70	0.24		—	0.59	0.26	
Presence of unstable carotid plaques n (%)	27 (51.9)	143 (51.1)	121 (46.0)		69 (52.7)	142 (46.0)	77 (49.7)	
OR (95% CI)	*	1.09 (0.22–3.66)	0.97 (0.38–2.50)	0.45	*	0.55 (0.14–2.18)	0.67 (0.20–2.26)	0.42
$p^{\dagger}$	—	0.56	0.59		—	0.39	0.51	

*Reference genotype is CC.

†p value for logistic regression analysis.

changes are systemic and not limited to cells infiltrating the vascular wall [11]. In contrast to these findings, we did not demonstrate an effect of VEGF/KDR polymorphisms on the presence of either plaques or unstable plaques, since no difference in genotype distribution was present.

In our study, the effect of either rs2071559 of KDR or rs2010963 on VEGF serum levels was demonstrated. These findings are in accordance with our previous studies in which subjects with recent MI history (up to 9 months after MI) were enrolled [13, 16, 25]. Moreover, increased plasma VEGF levels demonstrated in the stable phase after MI correlated with inflammation cytokines (IL-8 and IL-6), but not with atherosclerotic burden [25].

In contrast to the minor effect of the rs2071559 of KDR and the absence of the rs2010963 of the VEGF, an association of either rs2071559 or rs2010963 with MI has recently been

reported in Caucasians with T2DM [13, 16, 24]. Our present findings and previous reports are additional evidence that markers of carotid atherosclerosis and atherothrombotic events (i.e., MI) are most probably not regulated via similar genetical/biological mechanisms.

To conclude, in our study we demonstrated a minor effect of the rs2071559 of KDR on markers of carotid atherosclerosis (CIMT, sum of plaque thickness) in subjects with T2DM, whereas we failed to demonstrate an effect of tested polymorphism of the VEGF gene (rs2010963) on markers of carotid atherosclerosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Raziskava 2

POVEZAVA POLIMORFIZMOV ACE RS4646994 IN RS 4341 Z NAnapredeovanjem KAROTIDNE ATEROSKEROZE PRI SLOVENSKIH BOLNIKIH S SLADKORNO BOLEZNIJO TIPA 2

ASSOCIATION OF THE ACE RS4646994 AND RS4341 POLYMORPHISMS WITH THE PROGRESSION OF CAROTID ATHEROSCLEROSIS IN SLOVENIAN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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ORIGINAL ARTICLE

ASSOCIATION OF THE ACE rs4646994 AND rs4341 POLYMORPHISMS WITH THE PROGRESSION OF CAROTID ATHEROSCLEROSIS IN SLOVENIAN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT

The current study was designed to reveal possible associations between the angiotensin-converting-enzyme (*ACE*) gene polymorphisms (rs4646994 and rs4341) with markers of carotid atherosclerosis in patients with type 2 diabetes mellitus (T2DM) in a 4-year-long follow-up study. Five hundred and ninety-five T2DM subjects and 200 control subjects were enrolled. Genotyping of ACE polymorphisms was performed using KASPar assays, and ultrasound examinations were performed twice (at the enrollment and at follow-up). With regard to the progression of atherosclerosis in subjects with T2DM, statistically significant differences were demonstrated in the change of the sum of carotid plaques thickness for the rs4646994 polymorphism. We did not demonstrate an association between the tested polymorphisms (rs4646994 and rs4341) and either carotid intima media thickness (CIMT) or CIMT progression in a 3.8-year period. In our study, we demonstrated that subjects with T2DM with the DD genotype of the rs4646994 [ACE insertion/deletion (I/D)] polymorphism had faster progression of atherosclerosis in comparison to subjects with other genotypes.

Keywords: Angiotensin-converting-enzyme (*ACE*) gene polymorphism; Association study; Carotid atherosclerosis; Type 2 diabetes mellitus (T2DM).

INTRODUCTION

Type 2 diabetes mellitus (T2DM) represents a chronic illness characterized by the disability of the body to utilize glucose either because of insulin resistance in peripheral tissues or because of a decreased production of insulin by the pancreas [1]. Type 2 diabetes mellitus is known to promote the atherosclerotic process, which is characterized by endothelial dysfunction and by accumulation of foam cells and vessel wall inflammation. As the process continues, the narrowing of the vessel lumen occurs, leading to acute cardiovascular events [2].

The renin-angiotensin-aldosterone system is one of the main regulators of blood pressure having also other local (tissue-specific) roles [3]. Genetic polymorphisms in different parts of this system have previously been described to associate with various cardiovascular and other diseases, with the angio-

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tensin-converting-enzyme (ACE) insertion/deletion (I/D) polymorphism representing one of the most commonly studied polymorphisms that affects circulating ACE levels [3]. This polymorphism has recently been shown to be in linkage disequilibrium with another ACE polymorphism, rs4341 [3]; however, data about these two polymorphism and their possible association with carotid atherosclerosis in patients with diabetes mellitus are limited.

The present study was thus designed to investigate the association between polymorphisms of the *ACE* gene (rs 4646994 and rs4341) and markers of carotid atherosclerosis [carotid intima media thickness (CIMT), number of affected segments of carotid arteries and sum of plaques thickness] in patients with T2DM. The second aim was to see whether these two polymorphisms (rs4646994 and rs4341) affect progression of carotid atherosclerosis in a 4-year follow-up.

MATERIALS AND METHODS

In this cross-sectional study, 595 (338 males; 257 females) subjects with T2DM and 200 (92 males; 108 females) subjects without T2DM (control group) were enrolled as described previously [4]. The study protocol was approved by the Slovene Medical Ethics Committee (128/09/ 2010). After informed consent for participation in the study was obtained, a detailed interview was made.

All ultrasound examinations were performed by two experienced doctors blinded to the participants' diabetes status. The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured as described previously [4]. Plaques were defined as a focal intima-media thickening and divided according to their echogenic/ echolucent characteristics into five types as described previously [4]. The inter-observer reliability for carotid plaque characterization was found to be substantial (κ = 0.64, p <0.001).

Blood samples for biochemical analyses were collected as described previously [4]. The genomic DNA was extracted from 100 μL of whole blood using a FlexiGene DNA isolation kit, in accordance with the recommended protocol (Qiagen GmbH, Hilden, Germany). The ACE polymorphisms (rs4646994 and rs4341) were determined by a novel fluorescence-based competitive allele-specific polymerase chain reaction (PCR) (KASPar; Kbioscience Ltd., Hoddesdon, Hertfordshire, UK), assay. Details of the method used can be found at http://www.kbioscience. co.uk/.

Continuous variables were expressed as means ± standard deviations (SDs) if normally distributed, and as median (interquartile range) if asymmetrically distributed. Continuous clinical data were compared using an unpaired Student's *t*-test or analysis of variance (ANOVA) when normally distributed and the Mann-Whitney U test or the Kruskal-Wallis H test when asymmetrically distributed. The Pearson χ^2 test was used to compare discrete variables.

To determine the association of the *ACE* gene polymorphisms (rs4646994 and rs4341) with CIMT, a multiple linear regression analysis was performed. We used an additive model in which common allele homozygotes were coded as 1, heterozygotes as 2, and rare allele homozygotes as 3. All the regression models were adjusted for the presence of well-established cardiovascular risk factors. The results are presented as standardized β coefficients and p values for the linear regression and by odds ratios (ORs) and 95% confidence intervals (CIs) for the logistic regression. A two-tailed p value of less than 0.05 was considered statistically significant. A statistical analysis was performed using the Statistical Package for the Social Science (SPSS) software for Windows, version 20 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patients with T2DM had a greater waist circumference, higher fasting glucose and Hb A_{1c} levels compared to controls, whereas there were no statistically significant differences in age, body mass index (BMI), and systolic and diastolic blood pressure between patients with T2DM and control subjects (Table 1). Patients with T2DM had lower total, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol levels and higher triglyceride levels compared to controls (Table 1). Plasma levels of inflammatory markers (*i.e.,* high sensitive C-reactive protein (hs-CRP) were statistically significantly higher in patients with T2DM compared to controls (Table 1).

The genotype distributions both in patients with T2DM and controls were in the Hardy-Weinberg equilibrium for both *ACE* gene polymorphisms

Table 1. Baseline characteristics of subjects with type 2 diabetes mellitus and control subjects without type 2 diabetes mellitus.

Parameters	T2DM Subjects (n = 595)	Control Subjects (n = 200)	p Value
Age (years)	61.38 ± 9.65	60.07 ± 9.18	0.07
Gender: males females	 338 (56.8%) 257 (43.2%)	 92 (46.0%) 108 (54.0%)	**0.008**
Duration of T2DM (years)	11.25 ± 7.88	–	–
Cigarette smokers (%)	53 (8.9%)	34 (17.0%)	**0.002**
Waist circumference (cm)	108.65 ± 12.88	93.31 ± 13.18	**<0.001**
BMI (kg/m^2)	30.96 ± 4.74	27.90 ± 4.42	0.16
SBP (mmHg)	146.98 ± 19.98	143.30 ± 16.6	0.86
DBP (mmHg)	85.75 ± 11.62	84.70 ± 11.6	0.19
Fasting glucose (mmol/L)	8.04 ± 2.57	5.27 ± 0.87	**<0.001**
Hb A_{1c} (%)	7.89 ± 3.56	4.79 ± 0.29	**<0.001**
Total cholesterol (mmol/L)	4.70 ± 1.19	5.36 ± 1.08	**<0.001**
HDL cholesterol (mmol/L)	1.19 ± 0.35	1.43 ± 0.37	**<0.001**
LDL cholesterol (mmol/L)	2.63 ± 0.94	3.24 ± 0.98	**<0.001**
Triglycerides (mmol/L)	1.9 (1.2-2.7)	1.3 (0.9-1.9)	**<0.001**
hs-CRP (mg/L)	2.2 (1.0-4.3)	1.3 (0.8-2.7)	**<0.001**
CIMT (μm)	1013.0 ± 208.0	979.0 ± 141.0	0.03

Continuous variables are expressed as means ± SDs if normally distributed and as median (interquartile range) if asymmetrically distributed. Categorical variables are expressed as frequency (percentage). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; Hb A_{1c}: glycated hemoglobin (Hb); HDL: high density lipoprotein; LDL: low density lipoprotein; hs-CRP: high sensitivity C-reactive protein; CIMT: carotid intima media thickness.

[rs4646994: T2DM (genotype frequencies: II genotype 21.5%, ID genotype 46.4%, DD genotype 32.1%; $\chi^2 = 2.27$; $p = 0.13$) and controls (genotype frequencies: II genotype 24.0%, ID genotype 49.0%, DD genotype 27.0%; $\chi^2 = 0.07$; $p = 0.78$); rs4341: T2DM (genotype frequencies: GG genotype 28.1%, GC genotype 52.1%, CC genotype 19.8%; $\chi^2 = 1.44$; $p = 0.23$) and controls (genotype frequencies: GG genotype 23.5%, GC genotype 54.5%, CC genotype 22.0%; $\chi^2 = 1.63$; $p = 0.20$)]. No statistically significant differences in the ACE rs4646994 and rs4341 genotype distribution frequencies were observed between the T2DM patients and controls. Moreover, in our study, linkage disequilibrium between the two selected single nucleotide polymorphisms (SNPs) (rs4646994 and rs4341) was confirmed (d' =0.98 r^2=0.82).

Several parameters of carotid atherosclerosis, such as CIMT, number of involved segments, and sum of plaque thicknesses, were evaluated with regard to different genotypes of both ACE polymorphisms in subjects with T2DM at enrollment and after 3.8 years (Table 2). Moreover, the parameters of progression of atherosclerosis, *i.e.,* annual increase in CIMT, change in number of segments with plaques and change in the sum of carotid plaque thicknesses, were analyzed with univariate and multiple linear regression analyses (Tables 3 and 4). With regard to the progression of atherosclerosis in subjects with T2DM, statistically significant differences were demonstrated in the change of the sum of carotid plaque thickness for the rs4646994 polymorphism only (Table 3). Finally, according to the results of multiple linear regression analysis, faster progression of atherosclerosis was demonstrated in subjects with T2DM with the DD genotype of the rs 4646994 (ACE I/D) polymorphism in comparison with subjects with other genotypes (Table 4).

DISCUSSION

In this study, we demonstrated the effect of the DD genotype of the rs4646994 (ACE I/D) polymorphism on atherosclerosis progression in subjects with T2DM. Statistically significant differences in a 3.8-year observation period were found in the change in the sum of carotid plaques for the rs4646994 polymorphism. Our findings are in accordance with the findings of Saitou *et al.* [5], who on a cohort of 222

Table 2. Comparison of markers of carotid atherosclerosis in subjects with type 2 diabetes mellitus at the beginning and the end of the study with regard to the rs4646994 (angiotensin-converting-enzyme insertion/deletion) and rs4341 polymorphisms.

rs4646994 (ACE I/D)	**Enrollment**				**Endpoint**			
	II	**ID**	**DD**	***p* Value**	**II**	**ID**	**DD**	***p* Value**
Intima media thickness (μm)	998.0±147.0	1002.0±178.0	1012.0±178.0	0.62	1048.0±147.0	1054.0±128.0	1060.0±164.0	0.59
Involved segments (*n*)	2.65±1.49	3.04±1.71	2.66±1.44	0.12	4.48±1.75	4.62±1.68	4.71±1.76	0.68
Sum of plaque thickness (mm)	7.92±3.47	8.99±3.38	7.67±3.14	0.09	10.17±4.29	10.08±5.26	11.38±5.32	0.76
rs4341	**Enrollment**				**Endpoint**			
	GG	**GC**	**CC**	***p* Value**	**GG**	**GC**	**CC**	***p* Value**
Intima media thickness (μm)	1022.0±212.0	1002.0±217.0	1018.0±191.0	0.71	1065.0±155.0	1054.0±163.0	1051.0±164.0	0.62
Involved segments (*n*)	2.49±1.48	2.54±1.73	2.44±1.57	0.89	3.60±1.76	3.70±1.64	3.70±1.78	0.96
Sum of plaque thickness (mm)	7.77±4.50	8.02±4.42	7.53±4.77	0.74	9.38±5.34	9.16±5.16	8.72±4.56	0.88

ACE I/D: angiotensin-converting-enzyme insertion/deletion.

Table 3. Changes of markers of carotid atherosclerosis in subjects with type 2 diabetes mellitus between first the examination and the examination at the end of the study with regard to the rs4646994 (angiotensin-converting-enzyme insertion/deletion) and rs4341 polymorphisms.

rs4646994 ACE I/D	**II**	**ID**	**DD**	***p* Value**
Annual increase in CIMT (μm/year)	14.28 (5.35-26.83)	22.43 (16.73-32.19)	20.34 (10.53-33.65)	0.34
Δ Number of segments with plaques	3.0 (1.0-3.0)	1.0 (0.5-2.5)	2.0 (1.0-3.0)	0.42
Δ Sum of carotid plaques thickness (mm)	4.00 (2.30-5.30)	4.68 (3.30-7.60)	6.22 (3.90-8.10)	0.04
rs4341	**GG**	**GC**	**CC**	***p* Value**
Annual increase in CIMT (μm/year)	21.05 (14.28-33.65)	20.69 (16.55-24.26)	14.28 (10.71-20.08)	0.26
Δ Number of segments with plaques	2.0 (1.0-3.5)	2.0 (1.0-2.5)	3.0 (2.0-3.0)	0.49
Δ Sum of carotid plaques thickness (mm)	4.60 (3.40-7.90)	5.60 (4.35-8.55)	5.6 (2.60-6.90)	0.07

ACE I/D: angiotensin-converting-enzyme insertion/deletion. Annual increase in CIMT (carotid intima media thickness) was calculated as CIMT(beginning)-CIMT(endpoint)/follow-up in years. Change in number of plaques is expressed as number of segments with plaque at the end-point minus the number at the beginning. Sum of plaque thickness is calculated as the end sum minus the beginning sum. Data are expressed as median and range.

Table 4. Multiple linear regression analysis for association of rs4646994 (angiotensin-converting-enzyme insertion/deletion) with carotid atherosclerosis progression in patients with type 2 diabetes mellitus.

Parameters	**Δ CIMT/Year**		**Δ Number of Segments**		**Δ Sum of Plaque Thickness**	
	β	***p* Value**	β	***p* Value**	β	***p* Value**
A) rs4646994						
Hypertension (yes/no)	0.144	0.59	0.206	0.88	0.272	0.53
Systolic blood pressure	0.031	0.42	0.027	0.15	0.014	0.69
ID	0.141	0.49	0.116	0.51	0.845	0.26
DD	0.102	0.63	0.146	0.41	0.952	**0.04**

All models were adjusted to age, sex, smoking habits, serum levels of Hb A_{1c}, statin treatment and initial values of the dependent variables. Reference groups are homozygotes for the I allele.

T2DM patients reported faster progression of atherosclerosis in subjects with the D allele.

Moreover, in our study, we did not demonstrate an association between tested polymorphisms (rs4646994, rs4341) and either CIMT or CIMT progression in an almost 4-year period. In 1994, it was reported for the first time that ACE levels correlate with CIMT in healthy persons [6]. As the ACE I/D polymorphism is known to affect circulating ACE levels (subjects with the DD genotype having the highest serum ACE levels compared to subjects with other genotypes) [7], subsequent studies have already been conducted to identify a possible association of the ACE I/D genotype with atherosclerosis, both on general and diabetic populations, providing contradictory results [5-10]. Using smaller or larger cohorts of the general population, association of the ACE I/D polymorphism with carotid atherosclerosis was confirmed in some studies [6-8] and opposed by others [9]. Finally, large meta-analyses confirmed statistically significant association of ACE I/D with CIMT in the general population [10].

In studies that did not focus on the general population but on patients with T2DM, Kogawa *et al.* [11] in 1997, studied femoral and CIMT in healthy persons and patients with T2DM, and showed that *only* in T2DM patients was there a significant association of the D allele with higher CIMT. Since the study of Kogawa *et al.* [11], studies focusing on the association of CIMT and the ACE I/D polymorphism provided contradictory results, similar to studies conducted in the general population. In the diabetic heart study no association was found between ACE I/D and CIMT [12], similar to the study focusing on the offspring of patients with T2DM [13]. On the other hand, Sticchi *et al.* [14] reported a higher risk of carotid stenosis in D allele carriers, and Zhou *et al.* [15] reported higher lipid levels in older patients with T2DM carrying the D allele, which could promote atherosclerosis progression.

Certain limitations of our study should be noted. First is the moderate sample size of our study. However, all the participants were enrolled from an ethnically homogenous population, which minimizes possible biases from population stratification. Second, the results of our study may be affected by statin therapy, and antihypertensive agents, and these facts were not appreciated in the statistical analysis.

In conclusion, our study represents the first larger study focusing on the effect of two ACE polymorphisms (rs4646994 and rs4341) on the progression of carotid atherosclerosis in subjects with T2DM. We demonstrated that those subjects with T2DM with the DD genotype of the rs4646994 (ACE I/D) polymorphism had faster progression of atherosclerosis in comparison with subjects with other genotypes.

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Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Raziskava 3

POLIMORFIZEM GENA ARAHIDONSKE 5-LIPOOKSIGENAZE (ALOX5) IN POLIMORFIZEM GENA ARAHIDONSKE 5-LIPOOKSIGENAZE AKTIVACIJSKEGA PROTEINA (ALOX5AP) IN OZNAČEVALCI KAROITDNE ATEROSKLEROZE PRI BOLNIKIH S SLADKORNO BOLEZNIJO TIPA 2

ARACHIDONATE 5-LIPOXYGENASE (ALOX5) GENE POLYMORPHISM (RS12762303) AND ARACHIDONATE 5-LIPOXYGENASE ACTIVATING PROTEIN (ALOX5AP) GENE POLYMORPHISM (RS3802278) AND MARKERS OF CAROTID ATHEROSCLEROSIS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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Original Article

Arachidonate 5-lipoxygenase (ALOX5) gene polymorphism (rs12762303) and arachidonate 5-lipoxygenase activating protein (ALOX5AP) gene polymorphism (rs3802278) and markers of carotid atherosclerosis in patients with type 2 diabetes mellitus

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Abstract: Background: The study was designed to investigate the association between polymorphisms of the arachidonate 5-lipoxygenase (ALOX5) gene (rs12762303) and the arachidonate 5-lipoxygenase-activating proteingene (rs3802278), and markers of carotid atherosclerosis, such as carotid intima media thickness, the number of affected segments of carotid arteries and the sum of plaque thickness in patients with T2DM. Patients and methods: 595 T2DM subjects and 200 control subjects were enrolled. The carotid intima-media thickness (CIMT) and plaque characteristics (presence and structure) were assessed ultrasonographically. Biochemical analyses were performed using standard biochemical methods. Genotyping of the ALOX5 gene (rs12762303) and the ALOX5AP gene (rs3802278) was performed using KASPar assays. Results: In our study, we demonstrated an association between the rs3802278 and CIMT, and between the rs12762303 and coronary calcium score in subjects with T2DM. In our study, we did not demonstrate any association between tested polymorphisms (rs12762303 and rs3802278) and the sum of plaque thickness, the number of involved segments, hsCRP, the presence of carotid plaques or the presence of unstable carotid plaques. Conclusions: To conclude, in our study we demonstrated an association between the rs3802278 and CIMT, and between the rs12762303 and coronary calcium score in subjects with T2DM.

Keywords: Arachidonate 5-lipoxygenase, arachidonate 5-lipoxygenase activating protein, genetic polymorphism, association study, carotid atherosclerosis, type 2 diabetes mellitus

Introduction

Atherosclerosisis a chronic inflammatory disease [1]. Type 2 diabetes mellitus (T2DM) is considered a major epidemic of this century. T2DM is associated with an accelerated progression of atherosclerosis [2]. In patients with diabetes, cardiovascular complications are reported about 15 years earlier than in the population without T2DM [2-4].

Chronic, low-grade inflammation has been demonstrated to be involved in the pathogenesis of atherosclerosis in subjects at high risk to develop cardiovascular disease [5-8]. Moreover, genetic factors have long been known to modulate the risk of atherosclerosis and CVD, and they merit a search for the genes involved in the susceptibility to the atherosclerotic complications of T2DM [3, 9, 10]. Inflammation is involved in the pathogenesis of atherosclerosis,

Table 1. Baseline characteristics of subjects with T2DM and subjects without T2DM (control group)

	Subjects with T2DM N = 595	Control group N = 200	P
Age (years)	61.38 ± 9.65	60.07 ± 9.18	0.07
Male sex (%)	338 (56.8)	92 (46.0)	0.008
Diabetes duration (years)	11.25 ± 7.88	-	-
Cigarette smoking (%)	53 (8.91)	34 (17.0)	0.002
Waist circumference (cm)	108.65 ± 12.88	93.31 ± 13.18	< 0.001
BMI (kg/m^2)	30.96 ± 4.74	27.90 ± 4.42	0.16
SBP (mmHg)	146.98 ± 19.98	143.3 ± 16.6	0.86
DBP (mmHg)	85.75 ± 11.62	84.7 ± 11.6	0.19
Fasting glucose (mmol/L)	8.04 ± 2.57	5.27 ± 0.87	< 0.001
HbA1c (%)	7.89 ± 3.56	4.79 ± 0.29	< 0.001
Total cholesterol (mmol/L)	4.70 ± 1.19	5.36 ± 1.08	< 0.001
HDL cholesterol (mmol/L)	1.19 ± 0.35	1.43 ± 0.37	< 0.001
LDL cholesterol (mmol/L)	2.63 ± 0.94	3.24 ± 0.98	< 0.001
Triglycerides (mmol/L)	1.9 (1.2-2.7)	1.3 (0.9-1.9)	< 0.001
hs-CRP (mg/L)	2.2 (1.0-4.3)	1.3 (0.8-2.7)	< 0.001

Continuous variables were expressed as means ± standard deviations when normally distributed and as median (interquartile range) when asymmetrically distributed. Categorical variables were expressed as frequency (percentage). BMI-body mass index; SBP-systolic blood pressure; DBP-diastolic blood pressure; HbA1c-glycated haemoglobin; hs-CRP-high sensitivity C-reactive protein.

and the inflammatory process is triggered partially through the lipoxygenase pathway [1, 6, 11].

Leukotrienes (LTs) have been implicated as mediators, and potential therapeutic targets in the development of atherosclerosis. LTs are arachidonic acid-derived lipid mediators of inflammation. The initial step in the formation of LTs is catalyzed by 5-lipoxygenase (5-LO) in collaboration with the lipoxygenase-activating protein, subsequently leading to the formation of the LT family [11].

The mouse 5-LO gene, ALOX5, has been shown to contribute to the development of atherosclerosis [12]. In 2004, an association between *ALOX5* promoter polymorphism and an increased carotid intima media thickness was reported [13]. Dwyer and co-workers reported an increase in CIMT in subjects with two copies of the non-wild-type alleles of a tandem SP1 binding motif polymorphism in the *ALOX5* promoter compared with subjects who had two copies of the wild allele at this site [13]. The 5-lipoxygenase-activating protein, encoded by the ALOX5AP gene, likely acts as an arachidonic acid-binding and transfer protein to facilitate 5LO activity [14]. In several studies polymorphisms and haplotypes of ALOX5AP were reported to be associated with either myocardial infarction or ischemic stroke [15-19].

The present study was thus designed to investigate the association between polymorphisms of the ALOX5 gene (rs12762303) and the ALOX5AP gene (rs3802278), and markers of carotid atherosclerosis, such as carotid intima media thickness (CIMT), the number of affected segments of carotid arteries and the sum of plaque thickness in patients with T2DM.

Material and methods

This cross-sectional study included 595 subjects with T2DM and 200 subjects without T2DM (control group), as described above [20]. The study protocol was approved by the Slovene Medical Ethics Committee.

All ultrasound examinations were performed by two experienced doctors blinded to the participants' diabetes status. The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured, as described previously [20]. Plaques were defined as a focal intima-media thickening, and divided into 5 types according to their echogenic/echolucent characteristics, as described previously [20]. The inter-observer reliability for carotid plaque characterization was found to be substantial ($\kappa = 0.64$, $P < 0.001$).

In 215 out of 595 subjects with T2DM, coronary computed tomography angiography (CCTA) was performed for diagnostic purposes.

Blood samples for biochemical analyses were collected, as described previously [21]. The genomic DNA was extracted from 100 µL of whole blood using a Flexi Gene DNA isolation kit, in accordance with the recommended protocol (Qiagene GmbH, Hilden, Germany). Ge-

Table 2. Comparison of markers of carotid atherosclerosis in subjects with T2DM at the beginning of the study with regard to the ALOX5 gene (rs12762303) and the ALOX5AP gene (rs3802278) genotypes

	rs12762303			
	TT	TC	CC	P
Intima media thickness (μm)	1002 ± 190	1008 ± 196	917 ± 210	0.50
Number of involved segments	2.53 ± 1.59	2.48 ± 1.68	2.20 ± 1.80	0.89
Sum of plaque thickness (mm)	7.74 ± 4.40	8.29 ± 4.73	6.73 ± 4.60	0.556
hsCRP (mg/L)	3.26 ± 3.54	4.02 ± 3.59	3.00 ± 3.89	0.16
Presence of carotid plaques n (%)	339 (85.0)	152 (82.6)	9 (75)	0.89
Presence of unstable carotid plaques n (%)	183 (45.9)	95 (51.6)	7 (58.3)	0.63
Coronary calcium score	206 ± 282	429 ± 416	560 ± 215	0.005
	rs3802278			
	TT	TC	CC	p
Intima media thickness (μm)	982 ± 201	1023 ± 186	1037 ± 144	0.03
Number of involved segments	2.38 ± 1.51	2.63 ± 1.52	2.75 ± 1.54	0.24
Sum of plaque thickness (mm)	7.70 ± 4.40	8.23 ± 4.49	7.41 ± 4.68	0.53
hsCRP (mg/L)	3.38 ± 3.33	3.71 ± 3.84	3.18 ± 2.83	0.64
Presence of carotid plaques n (%)	261 (82.9)	189 (83.6)	50 (92.6)	0.28
Presence of unstable carotid plaques n (%)	145 (46.0)	120 (53.1)	20 (37.0)	0.06
Coronary calcium score*	269 ± 342	290 ± 347	162 ± 282	0.5

*Coronary computed tomography angiography (CCTA) was performed for diagnostic purposes in 215 out of 595 subjects with T2DM.

Table 3. Baseline characteristics of subjects with T2DM with unstable plaques and subjects with T2DM with stable plaques

	Subjects with T2DM with unstable plaques N = 190	Subjects with T2DM with stable plaques N = 140	P
Age (years)	63.42 ± 9.41	64.94 ± 8.83	0.44
Waist circumference (cm)	107.82 ± 13.24	110.86 ± 12.15	0.62
BMI (kg/m^2)	29.92 ± 4.85	28.90 ± 4.72	0.56
SBP (mmHg)	145.7 ± 17.7	144.3 ± 16.9	0.92
DBP (mmHg)	85.6 ± 11.5	84.9 ± 11.6	0.19
Fasting glucose (mmol/L)	7.98 ± 2.47	8.16 ± 2.65	0.58
HbA1c (%)	8.38 ± 2.56	7.63 ± 0.29	0.20
Total cholesterol (mmol/L)	4.82 ± 1.12	4.68 ± 1.20	0.27
HDL cholesterol (mmol/L)	1.15 ± 0.30	1.21 ± 0.36	0.10
LDL cholesterol (mmol/L)	2.66 ± 0.89	2.58 ± 0.99	0.46
Triglycerides (mmol/L)	2.43 (1.4-3.3)	2.34 (1.3-2.9)	0.65
hs-CRP (mg/L)	4.1 (0.8-6.3)	3.4 (1.1-5.7)	0.44
Coronary calcium score	294 ± 371	290 ± 305	0.90

Continuous variables were expressed as means ± standard deviations when normally distributed and as median (interquartile range) when asymmetrically distributed. Categorical variables were expressed as frequency (percentage). BMI-body mass index; SBP-systolic blood pressure; DBP-diastolic blood pressure; HbA1c-glycated haemoglobin; hs-CRP-high sensitivity C-reactive protein.

notyping of ALOX5 gene (rs12762303) and the ALOX5AP gene (rs3802278) was performed using KASPar assays. Details of the method used can be found on http://www.kbioscience.co.uk/.

Continuous variables were expressed as means ± standard deviations, when normally distributed, and as median (interquartile range) when asymmetrically distributed. Normality of the continuous variables was examined by the Kolmogorov-Smirnov test. Continuous clinical data were compared using an unpaired Student's *t* test or analysis of variance (ANOVA) when normally distributed and the Mann-Whitney U-test or the Kruskal-Wallis H-test when asymmetrically distributed. The Pearson X^2 test was used to compare discrete variables and to test whether the genotypes distribution is in Hardy-Weinberg equilib-

rium. A two-tailed *p*-value of less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 21 (SPSS Inc., Chicago, IL).

Results

Patients with T2DM had a greater waist circumference, higher fasting glucose and HbA1c levels compared to controls, whereas there were no statistically significant differences in age, gender distribution, BMI, systolic and diastolic blood pressure between patients with T2DM and control subjects (**Table 1**). Patients with T2DM had lower total, HDL and LDL cholesterol levels, and a higher triglyceride level compared to controls (**Table 1**). Plasma levels of inflammatory markers (i.e. hs-CRP and fibrinogen) were statistically significantly higher in patients with T2DM compared to controls (**Table 1**).

The genotype distributions in both patients with T2DM and controls were in Hardy-Weinberg equilibrium for the ALOX5 gene (rs12762303)-T2DM (genotype frequencies: TT genotype 67.1%, TC genotype 30.9%, CC genotype 2.0%; $\chi^2 = 3.08$; P = 0.08) and controls (genotype frequencies: TT genotype 69.0%, TC genotype 30.0%, CC genotype 1.0%; $\chi^2 = 2.69$; P = 0.1). The genotype distributions in both patients with T2DM and controls were in Hardy-Weinberg equilibrium for the ALOX5AP gene (rs3802278) polymorphism-T2DM (genotype frequencies: TT genotype 52.9%, TC genotype 38.0%, CC genotype 9.1%; $\chi^2 = 2.09$; P = 0.15) and controls (genotype frequencies: TT genotype 53.0%, TC genotype 39.0%, CC genotype 8%; $\chi^2 = 0.09$; P = 0.75).

The comparison of atherosclerosis parameters was performed with regard to different genotypes of both polymorphisms (rs12762303 and rs3802278) upon enrolment. In our study, we demonstrated an association between the rs3802278 and CIMT, and between the rs-12762303 and coronary calcium score in subjects with T2DM (**Table 2**). In our study, we did not demonstrate any association between tested polymorphisms (rs12762303 and rs-3802278) and the sum of plaque thickness, the number of involved segments, hsCRP, the presence of carotid plaques or the presence of unstable carotid plaques (**Table 2**).

The comparison of subjects with T2DM with unstable plaques and subjects with T2DM with stable plaques did not demonstrate any differences in lipid parameters, waist circumference, blood pressure, inflammatory parameters (hsCRP), or coronary calcium score (**Table 3**).

Discussion

In our study, we demonstrated an association between the rs3802278 of the ALOX5AP gene and CIMT, whereas the rs12762303 of the ALOX5 gene was not associated with CIMT in subjects with T2DM. Our findings are in accordance with some previous reports demonstrating that the variability in the *ALOX5* gene might be associated with CIMT [13, 22]. Burdon and co-workers, on the other hand, failed to demonstrate an association between another ALOX5 gene polymorphism (rs3780906) and CIMT in the Diabetes Heart Study [23]. Similarly, Assimes and co-workers demonstrated no association between the ALOX5 gene polymorphism (rs12762303) and CIMT either [24].

In our study, we did not demonstrate any association between tested polymorphisms (rs12-762303 and rs3802278) and the sum of plaque thickness, or the number of involved segments, or hsCRP, or the presence of carotid plaques, or the presence of unstable carotid plaques. Our findings indicate differential effects of the ALOX5/ALOX5AP genes on the markers of carotid atherosclerosis (i.e. CIMT) and on the markers of inflammation (i.e. hsCRP). Moreover, our findings are in accordance with a recently published report of van der Laan and co-workers who found no association between ALOX5/ALOX5AP polymorphisms and carotid plaque phenotypes [25]. Moreover, they found no association between ALOX5/ALOX5AP polymorphisms and either serum ALOX5 or ALOX5AP levels [24]. Additionally, Zhang and co-workers, who enrolled a total of 501 ischemic stroke patients and 497 healthy controls in their recent study, failed to demonstrate a statistically significant association between ALOX5AP rs4073259 and ischemic stroke in the Chinese Han population [19].

An interesting study demonstrating the importance of lipoxygenase was reported by Zhou and co-workers [26]. They performed immunohistological analysis of atherosclerotic plaques

with/without T2DM from 60 patients undergoing carotid endarterectomy [26]. They demonstrated increased 5-LO expression in diabetic plaques compared to non-diabetic plaques, and increased 5-LO expression was associated with increased MMP-2 and MMP-9 expression. They speculated that the over expression of 5-LO and LTB [6] in atherosclerotic plaques might promote an MMP-induced plaque rupture in diabetes [26]. Moreover, the over expression of 5-LO was reported in atherosclerotic symptomatic plaques in comparison with asymptomatic plaques [21].

In our study, we demonstrated an association between the rs12762303 of the ALOX5 gene and coronary calcium score in subjects with T2DM, whereas the rs3802278 of the ALOX5AP gene was not associated with coronary calcium score in subjects with T2DM. Similarly, Burdon and co-workers demonstrated an association between either the ALOX5 gene polymorphism (rs2115819) or the ALOXAP gene polymorphism (rs9506352) and coronary calcium score in the Diabetes Heart Study [23]. In a few other studies polymorphisms and haplotypes of ALOX5 and ALOX5AP were reported to be associated with myocardial infarction [15-18, 24, 27]. Several reports demonstrating no/minimal effect on subclinical carotid atherosclerosis and several reports on the association with MI might be additional evidence that markers of carotid atherosclerosis and atherothrombotic events (i.e. MI) are most probably not regulated via similar genetical/biological mechanisms [15-19, 22-25, 27].

Conclusion

To conclude, in our study we demonstrated an association between the rs3802278 and CIMT, and between the rs12762303 and coronary calcium score in subjects with T2DM. Our findings suggest that tested polymorphisms in the ALOX5/ALOX5AP genes play a minor role (if any) in the development of subclinical atherosclerosis in subjects with T2DM.

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Disclosure of conflict of interest

None.

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Raziskava 4

POLIMORFIZMA RS699 IN RS4762 ANGIOTENZIN GENA IN NAPREDOVANJE KAROTIDNE ATEROSKLEROZE PRI BOLNIKIH S SLADKORNO BOLEZNIJO TIPA II

POLYMORPHISMS RS699 AND RS4762 OF THE ANGIOTENSINOGEN GENE AND PROGRESSION OF CAROTID ATHEROSCLEROSIS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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Research Article Open Access

Polymorphisms rs699 and rs4762 of the Angiotensinogen Gene and Progression of Carotid Atherosclerosis in Patients with Type 2 Diabetes Mellitus

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Abstract

Background: The aim of the study was to investigate the association between two polymorphisms of the angiotensinogen (AGT) gene (rs699 and rs4762) and subclinical markers of carotid atherosclerosis in subjects with type 2 diabetes mellitus (T2DM).

Patients and methods: In this cross-sectional study 599 subjects with T2DM and 200 subjects without T2DM (control group) were enrolled. The CIMT and plaque characteristics (presence/absence and plaque thickness) on both the near and the far walls in the common carotid artery, bulb and internal carotid arteries, bilaterally were assessed ultrasonographically. After several years (3.8 ± 0.5 years), patients were re-assessed and changes in subclinical markers of carotid atherosclerosis were calculated. Polymorphisms rs699 and rs4762 of the AGT gene were genotyped by using allele-specific PCR (KASPar) assay.

Results: The highest increase in carotid plaque thickness was observed in homozygote carriers of the A allele, even after adjustment for confounding variables. Polymorphism rs699 did not affect the progression of CIMT in the increase of number of segments with plaques.

Conclusions: In the study we have found that the rs699 of the AGT gene is a potential genetic marker of carotid atherosclerosis progression (expressed as increase in carotid plaque thickness) in Slovenian patients with T2DM. It did not affect other ultrasonographic markers of carotid atherosclerosis progression. Polypmorphism rs4762 was not associated with carotid atherosclerosis progression in Slovenian patients with T2DM.

Keywords: Diabetes mellitus; Carotid aterosclerosis; Intima-media thickness; Angiotensinogen; rs699 polymorphism

Introduction

Cardiovascular and cerebrovascular diseases are leading causes of mortality in patients with diabetes mellitus [1,2]. Beside diabetes mellitus, some of the traditional risk factors, such as hypertension, hypercholesterolaemia, smoking and positive family history, contribute as well to the high prevalence of cardiovascular disease in subjects with diabetes mellitus [2].

Ultrasound examination of carotid arteries enables visualization of different aspects of atherosclerosis. Beside carotid intima-media thickness (CIMT), which is the most studied non-invasive phenotype of carotid atherosclerosis, we can also measure plaque thickness, total plaque area, plaque volume, determine type of plaques, and degree of stenosis [3]. CIMT is a good marker of early atherosclerosis and its progression [4]. It correlates well with cardiovascular risk factors and future cardiovascular events [5,6].

Some polymorphisms of the genes of the RAAS system have been so far associated with atherosclerotic cardiovascular disease [7,8]. Moreover, it has been reported that the renin angiotensin aldosterone system (RAAS) might affect CIMT and carotid atherosclerosis [9-13]; however, results are highly inconsistent.

The angiotensinogen (AGT) gene, an important gene of the RAAS system, is located in the long arm of chromosome 1 (gene locus 1q42-q43). The most studied AGT polymorphism, the rs699 (M235T) polymorphism, was reported to correlate with plasma AGT concentrations, whereas the highest levels of AGT are seen in the TT homozygotes [14-16]. There is still a limited number of studies focused on the association between the rs699 (M235T) and other AGT gene polymorphisms and markers of carotid atherosclerosis in the general population, and only one study in subjects with T2DM. Available data did not provide evidence of an association between the polymorphism of the genes in the RAS and either CIMT or the presence of carotid plaques in the general population, whereas only one study was performed in subjects with T2DM [8,9,17-20].

The aim of the study was to investigate the association between two polymorphisms of the angiotensinogen (AGT) gene (rs699 and rs4762) and subclinical markers of carotid atherosclerosis in subjects with T2DM.

Methods

Patients

In this prospective study 599 subjects with T2DM and 200 subjects without T2DM (control group) were enrolled.

Among patients with T2DM 399 were on statin therapy: 58.6% (234) received atorvastatin 20 mg per day, 22.5 %(90) received rosuvastatin 10 mg per day and 18.9% (75) received simvastatin 40 mg per day, while 196 were without hypolipemic therapy. They were selected among patients admitted to the diabetes outpatient departments of two general hospitals (Murska Sobota and Slovenj Gradec), and from cardiology outpatient department Medicor, Ljubljana, as described previously [21].

Ultrasonographic analysis

A high resolution B mode ultrasound analysis was performed using a portable ultrasound system, Toshiba Aplio SSA-700 (Toshiba Medical. Sys-tem Corp., Tokyo, Japan). All examinations were performed by two radiologists, blinded to the participants' diabetes status, as described previously [22]. The CIMT was measured at 3 sites along the 10 mm long segment of the far wall of the CCA free of plaques, in agreement with the carotid intima-media thickness consensus, as described previously [21,22]. The interobserver reliability for CIMT measurements was found to be substantial (κ= 0.74, $p < 0.001$) [22]. Moreover, plaques and type of plaques according to echogenic/echolucent characteristics were idefined and plaque thickness was measured in the common carotid artery, bulb and internal carotid artery, bilaterally, as described previously [22,23]. The interobserver reliability for carotid plaque characterization was found to be substantial (κ = 0.64, $p < 0.001$). After several years (3.8 ± 0.5 years), patients were re-assessed and subclinical markers of carotid atherosclerosis were checked again.

Biochemical analyses

Blood samples for biochemical analyses (total cholesterol, triglyceride levels, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol level, fasting blood glucose and glycated haemoglobin (HbA1c), and hsCRP) were collected, and all the biochemical analyses were determined in the hospital's accredited lab, as described previously [24].

	Subjects with T2DM	Control group	p
	n=595	n=200	
Age (years)	61.38 ± 9.65	60.07 ± 9.18	0.07
Male sex (%)	338 (56.8)	92 (46.0)	0.008
Diabetes duration (years)	11.25 ± 7.88	-	-
Cigarette smoking (%)	53 (8.91)	34 (17.0)	0.002
Waist circumference (cm)	108.65 ± 12.88	93.31 ± 13.18	<0.001
BMI (kg/m^2)	30.96 ± 4.74	27.90 ± 4.42	0.16
SBP (mm Hg)	146.98 ± 19.98	143.3 ± 16.6	0.86
DBP (mm Hg)	85.75 ± 11.62	84.7 ± 11.6	0.19
Fasting glucose (mmol/l)	8.04 ± 2.57	5.27 ± 0.87	<0.001
HbA1c (%)	7.89 ± 3.56	4.79 ± 0.29	<0.001
Total cholesterol (mmol/l)	4.70 ± 1.19	5.36 ± 1.08	<0.001
HDL cholesterol (mmol/l)	1.19 ± 0.35	1.43 ± 0.37	<0.001
LDL cholesterol (mmol/l)	2.63 ± 0.94	3.24 ± 0.98	<0.001
Triglycerides (mmol/l)	1.9 (1.2-2.7)	1.3 (0.9-1.9)	<0.001
hs - CRP (mg/l)	2.2 (1.0-4.3)	1.3 (0.8-2.7)	<0.001

Continuous variables were expressed as means ± standard deviations when normally distributed and as median (interquartile range) when asymmetrically distributed. Categorical variables were expressed as frequency (percentage). BMI - Body Mass Index; SBP - Systolic Blood Pressure; DBP - Diastolic Blood Pressure; HbA1c – Glycated Haemoglobin; hs-CRP - High Sensitivity C-Reactive Protein.

Table 1: Demographic and laboratory characteristics of subjects with T2DM and subject without T2DM (control group).

Genotyping

The genomic DNA was extracted from 100 µL of whole blood using a FlexiGene DNA isolation kit, in accordance with the recommended protocol (Qiagene GmbH, Hilden, Germany). The AGT rs699 and rs4762 polymorphisms were genotyped by novel fluorescence-based competitive allele-specific PCR (KASPar) assay (KBioscience Ltd). Details of the method used can be found at http://www.kbioscience.co.uk/.

Statistical analysis

Continuous variables were expressed as means ± standard deviations, and as median (interquartile range) when asymmetrically distributed was examined by the Kolmogorov-Smirnov test was used to examine the normality of the continuous variables. Continuous clinical data were compared using an unpaired Student's t test or analysis of variance (ANOVA) when normally distributed, and the Mann-Whitney U-test or the Kruskal-Wallis H-test when asymmetrically distributed. The Pearson X2 test was used to compare discrete variables and to test whether the genotypes distribution is in Hardy-Weinberg equilibrium. Moreover, pairwise linkage disequilibrium (LD; D` and $r^2$) between SNPs were calculated with Haploview software. rs699 and rs4762 were in complete LD (D`=1; r^2=0.21).

Pearson's correlation was performed to examine the association between independent variables.

A multivariate linear regression analysis was performed to determine the association of the AGT rs699 and rs4762 polymorphisms with the CIMT. To determine the association of the AGT rs699 and rs4762 polymorphisms with the presence of atherosclerotic plaques on the carotid arteries, a multivariate logistic regression analysis was performed. A two-tailed P value of less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 22 (SPSS Inc., Chicago, IL).

Results

The baseline clinical and biochemical characteristics of the study participants are shown in Table 1. There were no statistically significant differences in age, BMI and systolic and diastolic blood pressure between patients with T2DM and subjects without T2DM. Patients with T2DM had greater waist circumference, higher fasting glucose and HbA1c levels than the controls. Smoking prevalence was lower in patients with T2DM than in the control group. A comparison of the lipid parameters showed lower total, HDL, LDL cholesterol levels and a higher triglyceride level in patients with T2DM than in controls. Plasma levels of (hs-CRP was statistically significantly higher in patients with T2DM than in controls.

The AGT rs699 and rs4762 genotypes distribution and allele frequencies among the study subjects are shown in Table 2. The genotype distributions in both patients with T2DM and controls were compatible with Hardy-Weinberg expectations (rs699: T2DM: χ^2 = 0.09; p = 0.77; healthy controls χ^2 = 1.00; p = 0.32; rs4762: T2DM: χ^2 = 0.005; p = 0.94; healthy controls χ^2 = 2.98; p = 0.08). No statistically significant difference in the AGT rs699 and rs4762 genotypes distribution and allele frequencies was observed between patients with DM2 and the control group.

rs699	Subjects with T2DM n=595	Control group n=200	p
GG	123 (20.7)	37 (18.5)	0.74
GA	299 (50.3)	106 (53.0)	
AA	173 (29.0)	57 (28.5)	
Allele frequencies			
G	545 (45.8)	180 (45.0)	0.78
A	645 (54.2)	220 (55.0)	
rs4762			
GG	425 (71.4)	146 (73.0)	0.34
GA	156 (26.2)	46 (23.0)	
AA	14 (2.4)	8 (4.0)	
Allele frequencies			
G	1006 (84.5)	338 (84.5)	1
A	184 (15.5)	62 (15.5)	

Table 2: Genotype distribution of rs699 AGT and rs4762 in subjects with T2DM and control group.

Neither rs699 nor rs4762 had statistically significant impact on the CIMT at baseline (rs699: GG genotype vs. GA genotype vs. AA genotype = 980 ± 195 µm vs. 994 ± 187 µm vs. 1006 ± 196 µm, p=0.4; rs4762: GG genotype vs. GA genotype vs. AA genotype = 998 ± 193 µm vs. 1002 ± 187 µm vs. 1007 ± 189 µm, p=0.6)

rs699	GG	GA	AA	p
Annual increase in CIMT (µm/year)	12.41 (10.52-8.66)	17.14 (11.23-24.33)	21.05 (20.33-33.65)	0.59
Δ number of segments with plaques	2.0 (1.0-3.0)	2.3 (1.4-3.6)	2.5 (1.25-3.0)	0.16
Δ sum of carotid plaques thickness (mm)	5.45 (2.17-7.30)	6.30 (3.40-8.45)	7.30 (3.40-10.77)	0.03
Δ-changes in variables in observed time period expressed as percentage of the initial value				
CIMT - Carotid Intima Media Thickness				

Table 3: Changes of subclinical markers of carotid atherosclerosis in subjects with T2DM between first examination and control examination with regard to the genotypes of the rs699 polymorphism of the AGT.

Comparison of atherosclerosis progression (changes of markers of carotid atherosclerosis after 3.8 ± 0.5 years) showed the highest increase in carotid plaque thickness in subjects with T2DM carriers of the AA genotype of the rs699 (p=0.03). Although we observed the highest annual increase in CIMT and increase in number of sites with plaques in homozygotes for allele A, the differences were not statistically significant (Table 3).

In the present study we observed no statistically significant difference in atherosclerosis progression between carriers of different genotypes of the rs4762 polymorphism (Table 4).

rs4762	GG	GA+AA	p
Annual increase in CIMT (μm/year)	14.29 (10.71-21.05)	26.08 (15.43-33.65)	0.81
Δ number of segments with plaques	2.0 (1.5-3.0)	1.0 (0.5-2.5)	0.24
Δ sum of carotid plaques thickness (mm)	6.10 (3.50-7.60)	4.4 (2.45-6.90)	0.08
Δ-changes in variables in observed time period expressed as percentage of the initial value			
CIMT – Carotid Intima Media Thickness			

Table 4: Changes of subclinical markers of carotid atherosclerosis in subjects with T2DM between first examination and control examination with regards to the genotypes of the rs4762 polymorphism of the AGT.

As shown by multivariable linear regression analysis, the association of the AA genotype with highest increase in carotid plaque thickness remained significant even after adjustment for confounding variables (Table 5).

rs699 of the AGT gene*	ΔCIMT/year		Δnumber of segments		Δsum of plaques thickness	
	B	p	β	p	β	P
Hypertension (0=no; 1=yes)	0.082	0.87	0.124	0.82	0.264	0.56
Systolic blood pressure (mm Hg)	0.019	0.92	0.029	0.37	0.037	0.54
Genotype GA rs699	0.145	0.22	0.09	0.57	0.159	0.62
Genotype AA rs699	0.359	0.4	0.149	0.4	0.301	0.02
rs4762 of the AGT gene*						
Hypertension (0=no; 1=yes)	0.067	0.66	0.147	0.79	0.464	0.26
Systolic blood pressure (mm Hg)	0.078	0.62	0.035	0.28	0.046	0.31
GA+AA of the rs4762	0.473	0.6	-0.098	0.39	-0.058	0.08
All models were adjusted for age, sex, smoking, and the values of dependent variables at the enrollment in the study.						
* Reference groups for the rs699 and rs4762 are homozygotes for G allele.						

Table 5: Multivariate linear regression analysis for association of polymorphisms rs699 and rs4762 of the AGT gene with subclinical markers of carotid atherosclerosis progression in subjects with T2DM.

Discussion

In the study we confirmed an association between the AA genotype of the rs699 polymorphism of the AGT gene with the more rapid progression of carotid plaque thickness, whereas it was not associated with other markers of atherosclerosis progression. Contrary, polymorphism rs4762 of the AGT gene did not have a major impact on markers of carotid atherosclerosis progression in our population.

We speculate that the effect of the rs699 might be mediated via its effect on AGT serum levels, since the rs699 was found to correlate with plasma AGT concentrations [14]. There have been very few studies investigating the association between the AGT gene polymorphisms and markers of carotid atherosclerosis in either general population or in subjects with T2DM [8,9,17-20]. To our knowledge only one such study was performed in subjects with T2DM (European Americans and African Americans in the Diabetes Heart Study), and the rs699 did not appear to strongly influence subclinical cardiovascular disease (CIMT) in this population (20). This report is in accordance with our findings, since we also could not find significant impact of tested polymorphisms (rs699, rs4762) on CIMT at baseline. Moreover, Arnett et al. [9] reported no evidence that the rs699 of the AGT gene was associated with carotid intima-media thickness in middle-aged adults with no history of cardiovascular disease [7]. Similarly, Barley et al. [17] failed to demonstrate an association between the rs699 of the

AGT gene and either CIMT or cerebrovascular disease in one hundred consecutive Caucasian patients with internal carotid artery territory ischemia (TIA or stroke) [17]. Moreover, Sticchi et al also failed to report an association between carotid stenosis and the rs699 of the AGT gene [11]. Similarly, Losito et al. [12] failed to demonstrate an association between AGT gene polymorphisms (rs699, rs4762 - T174M) and either cerebrovascular disease or carotid stenosis in dialysis patients [10]. Contrary to several studies performed in either Caucasians or African Americans, Tabara et al. [11] reported an association between the rs699 of the AGT gene and CIMT in a middle-aged Japanese population [9].

Annual CIMT progression rate and the increase in total plaque thickness correlated differently with genetic and non-genetic risk factors for atherosclerosis development and progression, thus further supporting the hypothesis that CIMT and plaques are biologically distinct entities with different genetic backgrounds.

Conclusions

In conclusion, the rs699 of the AGT gene is a potential genetic marker of carotid atherosclerosis progression (expressed as increase in carotid plaque thickness) in Slovenian middle-aged patients (Caucasians) with T2DM. The rs699 of the AGT gene did not affect other ultrasonographic markers of carotid atherosclerosis progression

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Raziskava 5

POVEZAVA POLIMORFIZMOV (RS275651, RS931490 IN RS 5182) ANGIOTENZIN II TIP 1 RECEPTORJA Z NAPREDOVANJEM KAROTIDNE ATEROSKEROZE PRI KAVKAZIJSKI RASI S SLADKORNO BOLEZNIJO TIPA II

ASSOCIATION OF ANGIOTENSIN II TYPE 1 RECEPTOR POLYMORPHISMS (RS275651, RS931490 AND RS5182) WITH THE PROGRESSION OF CAROTID ATHEROSCLEROSIS IN CAUCASIANS WITH TYPE 2 DIABETES MELLITUS

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Poslano v objavo

Abstract

Hypothesis: The current study was designed to reveal possible associations between the angiotensin II type 1 receptor (AT1R) polymorphisms (rs275651, rs931490, and rs5182) with subclinical markers of carotid atherosclerosis in patients with type 2 diabetes mellitus (T2DM) in a four-year-long follow-up study.

Materials and methods: 595 T2DM subjects and 200 control subjects were enrolled. Genotyping of AT1R polymorphisms was performed using KASPar assays, and ultrasound examinations were performed twice (at the enrollment and at follow-up).

Results: Comparison of atherosclerosis progression (changes of markers of carotid atherosclerosis after 3.8 ± 0.5 years) in subjects with T2DM showed the highest increase in carotid plaque thickness in carriers of either the A allele of the rs275561 ($p = 0.02$) or the G allele of the rs931490 ($p = 0.02$), whereas in carriers of the T allele of the rs5182 we did not observe a significant effect on any marker of carotid atherosclerosis.

Conclusions: The rs275651 and the rs931490 of the *AT1R* gene may be considered as potential genetic markers of carotid atherosclerosis progression (expressed as increase in carotid plaque thickness) in Slovenian middle-aged patients with T2DM.

Keywords: diabetes mellitus, carotid atherosclerosis, intima-media thickness; angiotensin II type 1 receptor; polymorphisms; rs275651, rs931490, and rs5182

Introduction

It has been reported that the renin angiotensin aldosterone system (RAAS) and angiotensin II can play a causal role in the development and progression of atherosclerosis, along with the pathogenetic contribution of oxidative stress and inflammation (1-14). Angiotensin II has been demonstrated to induce oxidative stress, proinflammatory cytokine, and chemokine expression, endothelial cell death, infiltration of inflammatory cells into the vessel wall, and smooth muscle cell growth, migration, and proliferation (1,2,4). Although angiotensin II signals via seven transmembrane G-protein-coupled angiotensin II type 1 and 2 (AT1R and AT2R) receptors, it seems that the majority of its proatherogenic, proinflammatory, and pro-oxidant effects are mediated via AT1R (6-10). Angiotensin II is a potent inducer of oxidative stress, and several studies (11, 12) have established a central role for the Nox members of the NADPH oxidase family in smooth muscle cell migration and proliferation. The redox-sensitive transcription factors NF-κB and activator protein-1 are critical mediators of angiotensin II signaling (3). These results indicate that IL-18 amplifies the angiotensin II -induced, redox-dependent inflammatory cascades by activating similar promitogenic and promigratory signal transduction pathways (3). The angiotensin II /Nox1/IL-18 pathway may be critical in atherosclerosis.

Beside carotid intima-media thickness (CIMT), which is the most studied non-invasive phenotype of carotid atherosclerosis, we can evaluate with ultrasound examination of carotid arteries other subclinical markers of atherosclerosis, i.e. plaque thickness, total plaque area, plaque volume, degree of stenosis, and define type of plaques (15). CIMT is a good marker of early atherosclerosis and its progression, and it correlates well with cardiovascular risk factors and future cardiovascular events (16-18). Moreover, carotid atherosclerosis was reported to be a risk factor for future cardiovascular events (17).

The genetic predisposition to carotid atherosclerosis has been studied with respect to several candidate genes (19-26**)**. Because the RAAS plays a pivotal role in the regulation of blood pressure, as well as in cardiovascular remodeling, the genes encoding the components of RAAS have been the focus of much research (19, 20, 25, 26). Gene polymorphisms of the RAAS including angiotensin II type 1 receptor (AT1R), were investigated in relation to carotid atherosclerosis (19, 20, 25, 26). Moreover, it has been reported that the RAAS might affect CIMT and carotid atherosclerosis (27-32); however, results are highly inconsistent. Available data did not provide conclusive evidence of an association between the polymorphism of the genes in the AT1R and either CIMT or the presence of carotid plaques in the general population, whereas only one study was performed in subjects with T2DM (30, 32).

The present study was designed to investigate the association between three polymorphisms of the AT1R gene (rs275651, rs931490, and rs5182) and markers of carotid atherosclerosis (CIMT, number of affected segments of carotid arteries, and sum of plaques thickness) in patients with T2DM.

Material and methods

The study protocol was approved by the Slovene Medical Ethics Committee in September 2010 (115/07/2010 and 128/09/2010). After an informed consent for the participation in the study was obtained, a detailed interview was made. 595 subjects with T2DM and 200 subjects without T2DM (control group) were enrolled in this cross-sectional study. They were selected among patients admitted to the diabetes outpatient departments of two general hospitals from Slovenia (Murska Sobota, Slovenj Gradec), and from outpatient cardiology department Medicor, Ljubljana. Subjects in the control group were not allowed to have T2DM. Subjects with T2DM and control subjects were excluded if they had previous cardiovascular event (a myocardial infarction or a cerebrovascular stroke) or homozygous familial hypercholesterolaemia.

All ultrasound examinations were performed by two experienced doctors blinded to the participants' diabetes status. The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured. Plaques were defined as a focal intima-media thickening, and divided into 5 types according to their plaque characteristics, as previously described (33).

After several years, patients were re-assessed and markers of carotid atherosclerosis (CIMT, number of affected segments of carotid arteries, and sum of plaques thickness) were checked again.

Biochemical analyses

Blood samples for biochemical analyses: total cholesterol, triglyceride levels, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol level, fasting blood glucose and glycated haemoglobin (HbA1c), hsCRP and fibrinogen were collected. All the biochemical analyses were determined in the hospital's accredited lab.

Genotyping

The genomic DNA was extracted from 100μL of whole blood using a FlexiGene DNA isolation kit, in accordance with the recommended protocol (Qiagene GmbH, Hilden, Germany). The AT1R rs275651, rs931490, and rs5182 polymorphisms were determined were genotyped by KBioscience Ltd using their own novel fluorescence-based competitive allele-specific PCR (KASPar) assay. Details of the method used can be found at http://www.kbioscience.co.uk/.

Statistical analysis

Continuous variables were expressed as means ± standard deviations, when normally distributed, and as median (interquartile range) when asymmetrically distributed. Normality of the continuous variables was examined by the Kolmogorov-Smirnov test. Continuous clinical data were compared using an unpaired

Student's t test or analysis of variance (ANOVA) when normally distributed and the Mann-Whitney U-test or the Kruskal-Wallis H-test when asymmetrically distributed. The Pearson X2 test was used to compare discrete variables and to test whether the genotypes distribution is in Hardy-Weinberg equilibrium. Pearson's correlation was performed to examine the association between independent variables.

To determine the association of the AT1R rs275651, rs931490, and rs5182 polymorphisms with the CIMT a multivariate linear regression analysis was performed. To determine the association of the AT1R rs275651, rs931490, and rs5182 polymorphisms with the presence of atherosclerotic plaques on the carotid arteries, a multivariate logistic regression analysis was performed. We used an additive model in which common allele homozygotes were coded as 1, heterozygotes as 2 and rare allele homozygotes as 3. All the regression models were adjusted for the presence of well-established cardiovascular risk factors. The results were presented as standardized à coeficients and P-values for the linear regression and by odds ratios and 95 % CIs for the logistic regression. A two-tailed P value less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 20 (SPSS Inc., Chicago, IL).

Results

The baseline clinical and biochemical characteristics of the study participants are shown in Table 1. There were no statistically significant differences in age, gender distribution, BMI, systolic and diastolic blood pressure between patients with T2DM and controls. Patients with T2DM had greater waist circumference, a higher fasting glucose and HbA1c levels than the controls. Smoking prevalence was lower in patients with T2DM than in control group. A comparison of the lipid parameters showed lower total, HDL, LDL cholesterol levels and a higher triglyceride level in the patients with T2DM than in the controls. Plasma levels of inflammatory markers (hs-CRP and fibrinogen) were statistically significantly higher in patients with T2DM than in controls. No statistically significant difference in the AT1R rs275651, rs931490, and rs5182 genotypes distribution and allele frequencies was observed between the patients with DM2 and the healthy controls (data not shown). The genotype distributions in both patients with T2DM and controls were compatible with Hardy-Weinberg expectations (rs275561 T2DM: $\chi 2 = 0.001$; $p = 0.97$; controls $\chi 2 = 0.30$; $p = 0.58$; rs931490: T2DM: $\chi 2 = 0.21$; $p = 0.65$; controls $\chi 2 = 0.75$; $p = 0.39$; rs5182: T2DM: $\chi 2 = 2.49$; $p = 0.11$; controls $\chi 2 = 2.98$; $p = 0.08$).

Moreover, after 3.8 ± 0.5 years markers of carotid atherosclerosis were checked again. Comparison of atherosclerosis progression (changes of markers of carotid atherosclerosis after 3.8 ± 0.5 years) in subjects with T2DM showed the highest increase in carotid plaque thickness in carriers of either the A allele of the rs275561 ($p=0.02$) or the G allele of the rs931490 ($p = 0.02$), whereas in carriers of the T allele of the rs5182 we did not observe a significant effect on any marker of carotid atherosclerosis (Table 2).

As shown by multiple linear regression analysis, the association of either the A allele of the rs275561 or the G allele of the rs931490 with highest increase in carotid plaque thickness remained significant even after adjustment for confounding variables (Table 3).

Discussion

In the present study we confirmed for the first time the association of either the A allele of the rs275651 or the G allele of the rs931490 with highest increase in carotid plaque thickness in subjects with T2DM, whereas they were not associated with other markers of atherosclerosis progression (change in the number of affected segments of carotid arteries and the change in the sum of plaque thickness in carotid arteries).

In the present study we have demonstrated in Caucasians with T2DM that the rs275651and rs931490 of the AT1R are likely genetic markers for CIMT progression. Contrary, rs5128 of the AT1R gene did not have a significant impact on CIMT progression in our population. Our findings in subjects with T2DM are in accordance with the findings in general population in Japanese and Chinese studies (30,34,35). They reported an association between the AT1R gene polymorphism (A/C1166) and CIMT in middle-age Japanese and Chinese population. Our finding, however, are not in accordance with the findings of the Diabetes Heart Study enrolled similar sample size (620 European Americans and 117 African Americans), since they did not find an association between the AGT1R polymorphism and CIMT (36).

In our study we did not demonstrate a statistically significant effect of the tested gene polymorphisms (rs275651, rs931490, and rs5182) on markers of carotid atherosclerosis progression (change in the number of affected segments of carotid arteries and the change in the sum of plaque thickness in carotid arteries) in patients with T2DM. Our findings are in accordance with previous reports in general population in Caucasians and in Japanese (32,34,37). Contrary to findings in our study in subjects with T2DM and in general population (32,34,37). Moreover, in Caucasians (6000 subjects from the Rotterdam Study and more than 1000 subjects from the Rotterdam Scan Study) an association between the AT1R gene polymorphism (A/C1166 – CC genotype) and increased risk of silent brain infarction, but no effect on stroke (38). Additionally, AT1R polymorphism was reported to be affect the CAD/MI risk (19,20). CIMT is considered a separate phenotype from carotid plaques, and we presume that they have distinct genetic background. Moreover, they are most probably not regulated via similar pathogenetic mechanisms (18,39–42).

The strength of our cross-sectional study is rather large community-based sample of Caucasians with T2DM, and the detailed phenotypic characterization of the subjects with carotid atherosclerosis. Moreover, according to calculations the study was appropriately powered to detect differences in CIMT and carotid plaques thickness. A limitation of our study is the use of cross-sectional data in the analysis, restricting the possibility of causal inferences from our data.

Conclusions

In conclusion, the rs275651 and the rs931490 of the AT1R gene may be considered as potential genetic markers of carotid atherosclerosis progression (expressed as increase in carotid plaque thickness) in Slovenian middle-aged patients (Caucasians) with T2DM.

Conflicts of interest

There are no conflicts of interest existing.

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Table 1. Baseline characteristics of subjects with T2DM and healthy controls.

	Subjects with T2DM n = 595	Control group without T2DM n=200	**p**
Age (years)	62.39 ± 9.61	60.07 ± 9.18	0.008
Male sex (%)	338 (56.8)	92 (46.0)	**0.008**
Diabetes duration (years)	11.25 ± 7.88	-	**-**
Cigarette smoking (%)	53 (8.91)	34 (17.0)	**0.002**
Waist circumference (cm)	108.65 ± 12.88	93.31 ± 13.18	**<0.001**
BMI (kg/m2)	30.96 ± 4.74	27.90 ± 4.42	0.16
SBP (mm Hg)	146.98 ± 19.98	143.3 ± 16.6	0.86
DBP (mm Hg)	85.75 ± 11.62	84.7 ± 11.6	0.19
Fasting glucose (mmol/l)	8.04 ± 2.57	5.27 ± 0.87	**<0.001**
HbA1c (%)	7.89 ± 3.56	4.79 ± 0.29	**<0.001**
Total cholesterol (mmol/l)	4.70 ± 1.19	5.36 ± 1.08	**<0.001**
HDL cholesterol (mmol/l)	1.19 ± 0.35	1.43 ± 0.37	**<0.001**
LDL cholesterol (mmol/l)	2.63 ± 0.94	3.24 ± 0.98	**<0.001**
Triglycerides (mmol/l)	1.9 (1.2-2.7)	1.3 (0.9-1.9)	**<0.001**
hs CRP (mg/l)	2.2 (1.0-4.3)	1.3 (0.8-2.7)	**<0.001**

Continuous variables were expressed as means ± standard deviations when normally distributed and as median (interquartile range) when asymmetrically distributed. Categorical variables were expressed as frequency (percentage). BMI-body mass index; SBP-systolic blood pressure; DBP-diastolic blood pressure; HbA1c – glycated haemoglobin; hs-CRP- high sensitivity C-reactive protein.

Table 2. Changes of markers of carotid atherosclerosis in subjects with T2DM between first examination and control examination with regard to the genotypes of the rs275561 and rs931490 polymorphisms of the AGTR1.

rs275561	**TT**	**TA+AA**	**P**
Annual increase in CIMT (μm/year)	14.29 (11.74-21.38)	23.57 (17.06-33.65)	**0.02**
Δ number of segments with plaques	2.0 (1.5-3.0)	1.5 (0.25-2.75)	0.85
Δ sum of carotid plaques thickness (mm)	4.30 (1.20-7.95)	6.0 (3.62-8.27)	0.48
rs931490	**AA**	**AG+GG**	**P**
Annual increase in CIMT (μm/year)	14.46 (10.87-20.34)	23.22 (16.51-31.24)	**0.02**
Δ number of segments with plaques	2.0 (1.5-3.0)	1.5 (0.25-2.75)	0.68
Δ sum of carotid plaques thickness (mm)	4,2 (1.4-7.08)	5.90 (3.81-8.40)	0.74
rs5182	**CC**	**CT+TT**	**P**
Annual increase in CIMT (μm/year)	20.87 (14.28-25.34)	22.32 (10.88-28.64)	0.60
Δ number of segments with plaques	2.0 (0.5-3.0)	2.5 (1.25-3.75)	0.88
Δ sum of carotid plaques thickness (mm)	3.9 (1.3-7.25)	5.86 (2.26-8.25)	0.55

Δ-changes in variables in observed time period expressed as percentage of the initial value
CIMT – carotid intima media thickness

Table 3. Multiple linear regression analysis for association of rs275561, rs931490, and rs5182 of the AGTR1 gene with carotid atherosclerosis progression in patients with T2DM.

Parameter	ΔCIMT/year		Δnumber of segments		Δsum of plaques thickness	
	B	P	β	P	β	p
A:	**rs275561**					
Hypertension (yes/no)	0.120	0.94	0.161	0.24	0.224	0.55
Systolic blood pressure (mmHg)	0.024	0.87	0.032	0.81	0.019	0.45
TA+AA	0.212	**0.03**	-0.042	0.98	0.292	0.57
B:	**rs931490**					
Hypertension (yes/no)	0.223	0.48	0.142	0.32	0.273	0.53
Systolic blood pressure (mmHg)	0.008	0.58	0.020	0.88	0.022	0.53
AG+GG	0.213	**0.03**	-0.028	0.82	0.095	0.78
C:	**rs5182**					
Hypertension (yes/no)	0,047	0,74	0,148	0,29	0,648	0,20
Systolic blood pressure (mmHg)	0,020	0,88	0,053	0,71	0,035	0,25
CT+TT	0,086	0,57	0,062	0,47	0,226	0,17

Caption: All models are adjusted to age, sex, smoking, and initial values of the dependent variables. Reference group are homozygotes for the T allele (A), homozygotes for the A allele (B), and homozygotes for the C allele (C). CIMT – carotid intima media thickness

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Zaključek

Z opravljenimi raziskavami smo opredelili vlogo posameznih polimorfizmov genov renin-angiotenzinskega sistema, rastnih dejavnikov in lipooksigenazne poti pri razvoju ateroskleroze v populaciji slovenskih bolnikov s SB2.

Predvideli smo, da bodo testirani polimorfizmi genov vplivali na razvoj ateroskleroze vratnih arterij pri bolnikih s SB2.

Zaključki opravljenih raziskav:

1. Geni renin – angiotenzinskega sistema:
   a. Dokazali smo, da genotip DD polimorfizma rs4646994 v genu za AK vpliva na napredovanje ateroskleroze vratnih arterij pri bolnikih s SB2, kar se kaže v porastu seštevka plakov v vratnih arterijah.
   b. Nismo dokazali vpliva polimorfizmov rs4646994 in rs4341 na DIM vratnih arterij.
   c. Dokazali smo povezavo med polimorfizmom rs699 gena za AGT in debelino plakov v vratnih arterijah bolnikov pri bolnikih s SB2.
   d. Nismo dokazali povezave med polimorfizmom rs4762 v genu za AGT in označevalci ateroskleroze pri bolnikih s SB2.
   e. Dokazali smo povezavo med polimorfizmoma rs275651 in rs931490 gena za AT1R ter porastom seštevka debeline plakov v vratnih arterijah pri bolnikih s SB2.
2. Geni rastnih dejavnikov
   a. Dokazali smo povezavo med polimorfizmom rs2071559 gena za KDR in DIM ter seštevkom debeline plakov v vratnih arterijah pri bolnikih s SB2.
   b. Nismo uspeli dokazati povezave med polimorfizmom rs2010963 VEGF in debelino intime-medije ter ostalimi kazalci ateroskleroze vratnih arterij pri bolnikih s SB2.
   c. Nismo dokazali povezave med polimorfizmi genov VEGF in KDR ter prisotnostjo plakov vratnih arterij pri bolnikih s SB2.
3. Geni lipooksigenazne poti
   a. Dokazali smo povezavo med polimorfizmom rs3802278 gena za ALOX5AP in debelino intime-medije v vratnih arterijah pri bolnikih s SB2.
   b. Nismo uspeli dokazati povezave med polimorfizmom rs12762303 ALOX5 gena in označevalci ateroskleroze vratnih arterij pri bolnikih s SB2.

Z opravljenimi raziskavami in dobljenimi rezultati smo doprinesli k boljšemu razumevanju povezave med izbranimi polimorfizmi genov renin-angiotenzinskega sistema, rastnih dejavnikov in lipooksigenazne poti ter aterosklerozo vratnih arterij pri bolnikih s SB2.