Short Communication

Genetic Markers at *ANRIL*, *FTO* and *2q36.3* Locus in Czech Patients Undergoing Coronary Artery Bypass Graft Surgery

(polymorphism / FTO / ANRIL / 2q36.3 locus / coronary artery bypass surgery)

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Abstract: Coronary artery bypass graft (CABG) surgery is one of the most commonly performed operations worldwide. We compared genotype frequencies of three major cardiovascular disease (CVD)-associated genetic markers (*ANRIL*, *FTO* and *2q36.3* locus) between 753 patients who underwent CABG at the Institute for Clinical and Experimental Medicine (Prague, Czech Republic) and 2,559 controls from the Czech post-MONICA study. Subjects with at least one major A allele in the rs10757274 polymorphism (*ANRIL*) were more prevalent in patients after CABG than in the controls (81.7 % vs 72.7 %; OR [95 % CI] 1.67 [1.35-2.05]; P < 0.0001). In contrast, variants within the *FTO* gene (OR 0.87; 95% CI, 0.70-1. 09 in a TT vs. GG comparison, P = 0.24) and *2q36.3* locus (OR 1.16; 95% CI, 0.98-1.37 in a +A vs. CC comparison, P = 0.08) were not significantly associated with CVD in our study. Variants were not associated with anthropometric, biochemical, or clinical characteristics within the patient group. Our study suggests that patients with CABG are more commonly carriers of some but not all CVD-associated alleles.

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Abbreviations: BMI – body mass index, CABG – coronary artery bypass graft, CVD – cardiovascular disease, *FTO* – fat mass and obesity-associated gene, MIDCAB – minimally invasive direct coronary artery bypass, PCI – percutaneous coronary intervention, T2DM – type 2 diabetes mellitus.

Introduction

Coronary artery bypass graft (CABG) surgery is one of the most commonly performed operations worldwide (Weiss et al., 2006). The first hand-sewn coronary anastomosis is attributed to the Russian surgeon Vasilii I. Kolesov, who performed the first operation on February 25, 1964. In 1967, he published the results of a series of his first 12 patients (Kolesov, 1967). The most commonly used grafts for this procedure are *vena saphena magna* and *arteria thoracica interna*. The venous graft has a significantly shorter long-term patency compared to the arterial graft. The ten-year patency of the venous graft is about 50 % and for the left internal mammary artery about 90 %. Administration of acetylsalicylic acid and statins plays a positive role in the length of patency of the venous graft. Surgery is normally performed via longitudinal median sternotomy, but minimally invasive surgical approaches are becoming more popular. Most commonly, CABG is performed by anastomosing the left mammary artery to the *ramus interventricularis anterior* through anterior left-sided minithoracotomy (MIDCAB). This operation can alternatively be performed partially or fully robotically. The main advantage is a shorter recovery time and a reduction in the risk of complications of sternotomy healing. Myocardial revascularization can also be performed in a hybrid manner, combining a surgical approach (MIDCAB) and an interventional cardiology approach (PCI).

It is known that all cardiovascular disease (CVD) risk factors are partially determined by genetic predispositions. Further, there are some DNA markers associated with CVD that increase the risk of CVD without any association with the traditional risk factors (smoking, diabetes, dyslipidaemia, hypertension, or obesity). These markers have been detected through the genome-wide analysis approach (Uitterlinden, 2016). Most clinically relevant seem to be markers at positions 9p21 (*ANRIL*), 2q36.3, and the *FTO* polymorphism.

Variants within the *ANRIL* locus (OMIM acc. No. 613149) are likely the most important determinants of CVD across the populations (Palomaki et al., 2010; Kong et al., 2018). Variants associated with CVD are located within the long non-coding miRNA situated at the 9p21 locus, and it was estimated that one risky allele increases the risk of myocardial infarction by about 25 % and the risk is even higher in young subjects.

FTO ("fat mass and obesity-associated" gene; OMIM acc. No. 610966) was originally detected as an important determinant of body mass index (BMI) values (Frayling et al., 2007) and increased risk of type 2 diabetes mellitus (T2DM) (Scott et al., 2007). Later it was confirmed that variants within the first intron of the gene are independently associated with an increased risk of myocardial infarction (Doney et al., 2009; Hubacek et al., 2010; Lappalainen et al., 2011), as well as other non-communicable diseases (Dlouhá and Hubáček, 2012; Hubacek et al., 2012a; Hernández-Caballero and Sierra-Ramírez, 2015) such as Alzheimer's disease (Reitz et al., 2012) and health-associated phenotypes such as binge drinking (Hubacek et al., 2019) and eating behaviour (Hubáček et al., 2011; Abdella et al., 2019). Because of the extensive associations between the *FTO* variants and the wide list of individual phenotypes, the *FTO* gene locus (*RPGRIP1-l/FTO/IRX3*) has attracted a lot of attention. Despite intensive research, the understanding of the causal mechanism of how variants within this cluster affect the phenotypes remains unclear (Tung and Yeo, 2011; Tung et al., 2014).

Variants at the 2q36.3 locus are located within the "gene-free" area and were detected through the first wave of genome-wide association studies focused on CVD risk factors (Samani et al., 2007). Until now, the potential mechanism leading to the increased CVD risk is not known in detail for this locus, as the variant is located within the "gene-free" zone.

Our study was focused on the analysis of major genetic determinants of CVD in patients with coronary artery bypass surgery and comparison of the prevalence of individual genotypes with healthy controls.

Material and Methods

Patients

Altogether 753 patients (aged 59.7 ± 9.0 years) who underwent CABG at the Institute for Clinical and Experimental Medicine between 1st January, 1975 and 31st December, 2014 were included in the study. The study cohort consists of the most polymorbid of a total of 10,000 CABG patients (Kačer et al., 2020). Patients underwent cardiac surgery for severe ischaemic heart disease that could not be managed with medication alone. All patients went through a standard examination process according to the recommendations valid at the time of operation.

Controls

The controls consisted of a sample of 1,191 males (aged 49.0 ± 10.7 years at the time of examination) and 1,368 females (aged 48.8 ± 10.6 years at the time of examination) from the Czech Post-MONICA study (Tunstall-Pedoe et al., 2003; Hubacek et al., 2017; Cifková et al., 2020).

The study protocol was approved by the Ethic Committee of the Institute for Clinical and Experimental medicine and Thomayer Hospital and is in agreement with the Helsinki declaration of 1975 (protocol ID – 1049/15). All examined subjects signed informed consent with participation in the study.

Anthropometrical and biochemical parameters

Cholesterol, triglycerides, creatinine, albumin and glucose (in fasting plasma) were assessed using conventional enzymatic methods (reagents from Roche Boehringer Mannheim Diagnostics and Hoffmann-La Roche, Basel, Switzerland). Echocardiographic control examinations were performed using an Affiniti 70G instrument (Philips, Amsterdam, The Netherlands) in standard projections in a routine echocardiography laboratory. Diastolic and systolic blood pressures were measured after 10 min in a sitting position as an average of three readings on the right arm with an automated blood pressure unit.

Genetic analyses

DNA was isolated from frozen (−20 ºC) uncoagulated (EDTA) blood using a standard "salting-out" method (Miller et al., 1988). Genetic variants were analysed with PCR-RFLP in a 25-µl volume (Hubáček et al., 2015) in PCR device DYAD PTC-220 (MJ Research, Reno, NV, USA). All PCR chemicals and restriction enzymes were purchased from Fermentas International Inc., Burlington, Ontario, Canada.

The rs2943634 variant at *2q36.3* was examined using oligonucleotides 5´ aaa gca agc aca tct gtg gct gta c and 5´ tac act tga aaa ttg tag ttg ctc c, and the PCR product (150 bp) was cut with 5 units of the *Bsp*1407I restriction enzyme. For the rs10757274 variant (9p21 locus, *ANRIL*), oligonucleotides 5´ttg ctt ggt aga tct tcc tcc atc cct t and 5´ttc cca gat gca ctg tat tgt ttg cct tac were used, and the PCR product (225 bp) was cut using 5 units of the *Bsm*AI restriction enzyme. DNA fragments with *FTO* rs17817449 polymorphisms were amplified using oligonucleotides 5` ggt gaa gag gag gag att gtg taa ctg g and 5` gaa gcc ctg aga agt tta gag taa att ggg, and the PCR product (198 bp) was cleaved with restriction enzyme *Alw*NI.

For more protocol details, see Hubacek et al., 2012b, 2015, 2016. Restriction fragments were separated in 12% polyacrylamide gel using the MADGE technique (Day and Humphries, 1994) in a Tris-EDTA buffer.

Statistical analyses

χ^2 (http://www.physics.csbsju.edu/cgi-bin/stats/contingency_form.sh?nrow=2&ncolumn=3) and OR (95% CI) (http://www.hutchon.net/ConfidOR.htm) were calculated using freely available online tools. Genotypes were compared in dominant (AA+Aa vs. aa), co-dominant (AA vs. Aa vs. aa) and recessive (AA vs. Aa+aa) models (www.socscistatistics.com). As there were no significant sex-dependent differences within the patients and controls, males and females were pooled together for the comparisons. Potential genotype/phenotype associations were analysed using ANOVA.

Results and Discussion

General characteristics of the examined patients and controls are summarized in Table 1. As expected, patients were significantly older than controls ($P < 0.001$). The achieved genotyping success (call rate) varied between 96.9 % and 99.5 %.

As described before (Hubacek et al., 2009, 2012a, b, 2016), there was no association between the examined variants and traditional risk factors of CVD in the controls with the exception of *FTO*, which was associated with BMI (Hubacek et al., 2009) and increased prevalence of T2DM (Hubacek et al., 2018). In the patients, no associations between the available phenotypes and examined genotypes were detected (not shown in detail).

For the polymorphism within the *ANRIL* locus, carriers of at least one A allele were significantly more prevalent in the patients than in the controls (81.7 % vs 72.7 %; OR [95% CI] 1.67 [1.35–2.05]) (Table 2). This result is in agreement with a wide list of results published previously (Palomaki et al., 2010; Hubáček et al., 2012b; Vakalis et al., 2014; Kong et al., 2018), albeit on differentially defined patients. Interestingly, in a large multicentre study (Patel et al., 2019), the same polymorphism was not associated with increased cardiovascular mortality among patients with established congenital heart disease at baseline.

In contrast, genotype or allelic frequencies of the variants within the *FTO* gene ($P = 0.24$; OR [95% CI] 0.87; [0.70–1. 09] for TT vs. GG comparison) and 2q36.3 ($P = 0.08$; OR [95% CI] 1.16; [0.98–1.37] in a +A vs. CC comparison) locus were not significantly different between the patients and controls in any of the models of comparisons (Table 2). This is a rather surprising result, especially in the case of the *FTO* gene, as the *FTO* polymorphisms were associated with increased risk of CVD in a vast majority of studies performed on Caucasians (for example, Doney et al., 2009; Lappalainen et al., 2011; Hubacek et al., 2016). The gene encodes a protein with potential demethylase activity, suggesting a potential epigenetic regulatory function of the gene product (Mauer et al., 2019). It is possible that the relatively high age of our subjects could mimic the differences.

Despite the relatively high OR for CVD in the original GWAs study (Samani et al., 2007), the association between CVD and rs2943634 at the 2q36.3 locus was later detected in some but not all studies (Muendlein et al., 2009; Vakalis et al., 2014; Hubacek et al., 2016). In contrast to *FTO* and *ANRIL*, this locus did not get a lot of attention. This is likely because of the so far unknown potential biological functionality of this SNP. We can just speculate that this polymorphism could be, similarly as the two above-mentioned SNPs, involved in some way in CVD-associated epigenetic mechanisms.

In our study, we found an expected trend: the CC homozygotes were slightly more prevalent in patients than in the controls. The borderline for non-significance observed in our study suggests a relatively low power of this finding, as the number of the examined patients is below 1,000 subjects.

Table 1. General characteristics of the examined subjects

	Patients	**Controls**
Number	753	2, 559
Age (years)	59.7 ± 9.0	48.8 ± 10.7
Females (%)	54.6	53.5
Total-C (mmol/l)	5.50 ± 2.46	5.78 ± 1.11
LDL-C (mmol/l)	3.41 ± 1.17	3.51 ± 1.00
Triglycerides (mmol/l)	2.04 ± 1.35	1.82 ± 1.35
Smoking prevalence (%)	38.6	28.8
T2DM (%)	12.2	5.2
Glucose (mmol/l)	5.80 ± 3.96	5.28 ± 1.18
Hypertension (%)	41.2	37.0
LVEF (%)	58.3 ± 11.6	n.a.
Albumin (g/l)	37.4 ± 6.04	n.a.
Creatinin (μmol/l)	88.7 ± 12.5	n.a.

LDL-C – low-density lipoprotein cholesterol, LVEF – left ventricular ejection fraction, total-C – total cholesterol

Table 2. Genotype distributions of the examined polymorphisms within the patients after CABG and controls

2q36.3 rs2943634	Controls		Patients		OR	P¶	P*
	N	%	N	%	Crude		
CC	970	38.7	317	42.3	1.00		0.08[&]
CA	1176	47.0	339	45.3	0.89 (0.75–1.06)	0.16	0.16[#]
AA	358	14.3	93	12.4	0.79 (0.61–1.03)	0.08	0.19[§]
P for differences in the numbers of alleles						0.06	

9p21 rs10757274	Controls		Patients		OR	P¶	P*
	N	%	N	%	Crude		
GG	687	27.3	136	18.4	1.00		0.0005[&]
AG	1242	49.3	400	54.0	1.63 (1.31–2.02)	0.0005	0.0001[#]
AA	589	23.4	205	27.7	1.76 (1.38–2.24)	0.005	0.02[§]
P for differences in the numbers of alleles						0.0001	

FTO rs17817449	Controls		Patients		OR	P¶	P*
	N	%	N	%	Crude		
TT	426	17.3	144	19.2	1.00		0.22[&]
GT	1224	49.6	362	48.3	0.87 (0.70–1.09)	0.24	0.47[#]
GG	817	33.1	243	32.4	0.88 (0.69–1.12)	0.29	0.73[§]
P for differences in the numbers of alleles						0.37	

P¶ is calculated for comparison as for OR; P* is calculated for dominant[&], co-dominant[#] and recessive[§] models of comparisons.

Generally, surgical mortality of CABG is low, at around 2 % (El Bardissi et al., 2012). Compared to the rest of the population, the mortality in CABG patients is higher in the first 30 days after surgery due to a higher risk of myocardial infarction, heart failure and stroke. It then remains the same and increases again 8–10 years after the surgery (Adelborg et al., 2017). Thus, we do not expect that the surgical mortality will significantly affect the outcomes of our study.

In Western Europe, 62 out of 100,000 individuals in the general population undergo this operation (OECD, 2009; Head et al., 2017) per year. In the Czech Republic, 3,318 isolated CABG operations and 1,435 CABG operations combined with other cardiac surgical procedures were performed in 2018. The annual numbers of CABG procedures have been stable over the last eight years (NKR: NZIS Report No. R01 (12/2019)).

Taken together, although we did not achieve the significance described previously, the genotype frequencies of the examined polymorphisms are within the group of CABG patients similar to the Czech population controls/patients with acute coronary syndrome (Hubacek et al., 2010, 2012a, b, 2016).

In our group of polymorbid CABG patients, we did not detect any associations with biochemical and anthropometrical CVD risk factors and the examined SNPs. This is in agreement with previous studies both on general populations and CVD patients and further underlines that the physiological significance of these nucleotide changes needs to be studied.

Our study suggests that patients with CABG are more commonly carriers of some but not all CVD-associated alleles. This suggests high genetic heterogeneity between CABG patients and subjects suffering from myocardial infarction.

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Conflict of interest statement

Authors declare no conflict of interest.

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