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Joint effect of insulin signaling genes on cardiovascular events and on whole body and endothelial insulin resistance

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ABSTRACT

Objective: Insulin resistance (IR) and cardiovascular disease (CVD) share a common soil. We investigated the combined role of single nucleotide polymorphisms (SNPs) affecting insulin signaling (*ENPP1* K121Q, rs1044498; *IRS1* G972R, rs1801278; *TRIB3* Q84R, rs2295490) on CVD, age at myocardial infarction (MI), *in vivo* insulin sensitivity and *in vitro* insulin-stimulated nitric oxide synthase (NOS) activity.
Design and setting: 1. We first studied, incident cardiovascular events (a composite endpoint comprising myocardial infarction-MI, stroke and cardiovascular death) in 733 patients (2186 person-years, 175 events). 2. In a replication attempt, age at MI was tested in 331 individuals. 3. OGTT-derived insulin sensitivity index (ISI) was assessed in 829 individuals with fasting glucose <126 mg/dl. 4. NOS activity was measured in 40 strains of human vein endothelial cells (HUVECs).
Results: 1. Risk variants jointly predicted cardiovascular events (HR = 1.181; $p = 0.0009$) and, when added to clinical risk factors, significantly improved survival C-statistics; they also allowed a significantly correct reclassification (by net reclassification index) in the whole sample (135/733 individuals) and, even more, in obese patients (116/204 individuals). 2. Risk variants were jointly associated with age at MI ($p = 0.006$). 3. A significant association was also observed with ISI ($p = 0.02$). 4. Finally, risk variants were jointly associated with insulin-stimulated NOS activity in HUVECs ($p = 0.009$).
Conclusions: Insulin signaling genes variants jointly affect cardiovascular disease, very likely by promoting whole body and endothelium-specific insulin resistance. Further studies are needed to address whether their genotyping help identify very high-risk patients who need specific and/or more aggressive preventive strategies.

1. Introduction

Cardiovascular disease (CVD) is highly prevalent [1] and imposes a tremendous burden to health care systems as well as to

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patients and their families. Genetic and environmental factors both contribute to CVD. Genes predisposing to CVD are mostly unknown [2] with only few clues about their identities being provided by recent genome-wide association studies (GWAS) [3–9]. Insulin resistance, and the related cluster of abnormalities, is pathogenic for CVD [10,11]. Since also insulin resistance is under genetic control [12,13], genes that contribute to impaired insulin sensitivity may also be involved in shaping cardiovascular risk [13].

Several variants harbored by genes involved in the modulation of insulin action have been so far described. Among these, there are three single nucleotide polymorphism (SNPs) of insulin signalling regulating genes, namely rs1044498 (i.e. *ENPP1* K121Q), rs1801278 (i.e. *IRS1* G972R) and rs2295490 (*TRIB3* Q84R). These are the only nonsynonymous SNPs whose ability to affect the insulin signaling pathway has been thoroughly characterized *in vitro* not simply in transfected cell lines but also in human cells naturally carrying them [14–21]. In addition, several [22–26], although not all [27], *in vivo* studies have reported that each of these SNPs is associated with human insulin resistance. Finally, in human endothelial cells these SNPs have been reported to inhibit insulin-stimulated nitric oxide (NO) production [16,18,20], a potent vasodilator whose deficiency is an early step in the development of atherosclerosis [28]. Of note in this specific context, *ENPP1* K121Q has been also reported to predict major cardiovascular events [29]. So, these three SNPs represent a unique model perfectly suited to answer the question of whether a progressive impairment of the insulin signaling pathways, as a consequence of their combined effect, plays a role in modulating the risk insulin resistance-related alterations.

Accordingly, our present aim was to investigate whether these three functional SNPs (with risk variants being *ENPP1* Q121, *IRS1* R972 and *TRIB3* R84, respectively) exert a combined effect on major cardiovascular events in high-risk individuals and whether in these patients such genetic information improves the ability to predict CVD. In addition, in order to unravel the biology underlying the joint associated effect of the 3 risk variants on CVD we in fact observed, their combined roles on insulin action both *in vivo* (i.e. on glucose disposal) and *in vitro* (i.e. on NO production by cultured human endothelial cells) was also investigated.

2. Methods

2.1. Study samples and designs

Salient features of study subjects and designs regarding the prospective analysis on incident cardiovascular events, the cross-sectional association with age at myocardial infarction (MI) and the cohort study on insulin sensitivity are described into details in the online Supplemental Methods (i.e. “Study participants and design” section). Methods for performing studies on NO synthase (NOS) activity in human vein endothelial cells (HUVECs) are described in the same section of online Supplemental Methods.

All four study protocols were approved by the local institutional review boards and performed according to the Helsinki Declaration. Written informed consent was obtained from each study participant.

2.2. Genotyping

DNA was extracted from whole blood or HUVECs by standard methods. Genotyping of the *ENPP1* K121Q (rs1044498) *IRS1* G972R (rs1801278) and *TRIB3* Q84R (rs2295490) polymorphisms was performed by TaqMan allele discrimination (assays C_1207994_20, C_2384392_20 and C_16190162_10 respectively, Applied Biosystems, Forster City, CA) on the HT7900 platform (Applied Biosystems). Genotyping quality was assessed by including in each 96 wells plate positive controls with known genotypes (i.e. homozygous for the major allele, heterozygous or homozygous for the minor allele, previously evaluated for each SNP of interest by direct Sanger sequencing, each in duplicates). The agreement rate was >99% for each SNP assay. Genotype distribution was in Hardy–Weinberg equilibrium (HWE) in all study samples.

2.3. Statistical analysis

Patients’ characteristics are reported as mean and standard deviation (SD) for continuous variables and frequencies and percentages for categorical ones.

Deviations from HWE of each single SNP were investigated by exact χ^2 test.

The association between each single SNP and any given phenotype of interest was assessed according to the additive model of inheritance.

The association between the three SNPs considered in a combined fashion and any given phenotype of interest, was assessed by creating a weighted genotype risk score (GRS); this was obtained by summing the risk alleles from each SNP weighted by their estimated effect sizes, using regression coefficient as appropriate, on each outcomes of interest from current data. To facilitate results interpretation, each individual’s GRS was divided by the maximum observed GRS and multiplied by 6 (i.e. the maximal theoretical number of risk alleles). Each GRS weights were estimated following a bootstrap approach with 10,000 re-samplings with replacement in order to reduce the risk of too optimistic discoveries.

In prospective studies, time-to-event analysis was conducted by means of Cox proportional hazards regression models and reported as hazard ratios (HR) along with their 95% confidence interval (CI). Time variable was defined as the time between enrollment date and the date of any of the three major cardiovascular events comprised in our composite end-point. For subjects who did not experience any cardiovascular event, time variable was defined as the time between the enrollment date and the date of the last available clinical follow-up. The assumption of proportionality of the hazards was tested by using scaled Schoenfeld residuals.

At the time of study design, we calculated that our prospective study on incident cardiovascular events had 80% power at a 0.05 significance level (type I error $-\alpha$) to detect a HR $= 1.16$, for each CV-GRS unit in the whole sample ($n = 733$).

Details on addressing predicted risk probabilities, models’ discrimination, reclassification improvement are given in the online Supplemental Methods (i.e. “Statistical analysis” section).

In cross-sectional studies, multivariate logistic regression analysis was used to test for association with dichotomous outcomes and results were reported as odds ratios with 95% confidence intervals. One-way analysis of variance (ANOVA) or covariance (ANCOVA; i.e. in multivariate models) was used to test for association with continuous traits.

Details about analyses on pooled samples also are given in the online Supplemental Methods (i.e. “Statistical analysis” section).

Data on insulin-stimulated NOS activity in HUVECs were analyzed as percentage increase of insulin-stimulated NOS activity over basal, unstimulated value for each cell line.

Normal distribution was checked using Kolmogorov–Smirnov and Shapiro–Wilk tests as well as by graphical inspection of nonparametric kernel density estimation and Q–Q plot. Log-transformation was used for suspicious nonnormal distributed variables.

A p-value smaller than 0.05 was considered as significant. All analyses were performed using SAS Release 9.1 (SAS Institute, Cary, NC).