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Short Communication

Gene–environment and gene–gene interactions of specific MTHFR, MTR and CBS gene variants in relation to homocysteine in black South Africans

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ABSTRACT

The methylenetetrahydrofolate reductase (MTHFR), cystathione-β-synthase (CBS) and methionine synthase (MTR) genes interact with each other and the environment. These interactions could influence homocysteine (Hcy) and diseases contingent thereon. We determined single nucleotide polymorphisms (SNPs) within these genes, their relationships and interactions with total Hcy concentrations within black South Africans to address the increased prevalence of diseases associated with Hcy. The MTHFR 677 TT and MTR 2756 AA genotypes were associated with higher Hcy concentrations (16.6 and 10.1 μmol/L; $p < 0.05$) compared to subjects harboring the MTHFR 677 CT/CC and the MTR 2756 AG genotypes (10.5, 9.7 and 9.5 μmol/L, respectively). The investigated CBS genotypes did not influence Hcy. We demonstrated interactions between the area of residence and the CBS T833C/844ins68 genotypes ($p = 0.005$) so that when harboring the wildtype allele, rural subjects had significantly higher Hcy than their urban counterparts, but when hosting the variant allele the environment made no difference to Hcy. Between the CBS T833C/844ins68 or G9276A and MTHFR C677T genotypes, there were two-way interactions ($p = 0.003$ and $= 0.004$, respectively), with regard to Hcy. Subjects harboring the MTHFR 677 TT genotype in combination with the CBS 833 TT/homozygous 844 non-insert or the MTHFR 677 TT genotype in combination with the CBS 9276 GA/GG displayed higher Hcy concentrations.
Therefore, some of the investigated genotypes affected Hcy; residential area changed the way in which the CBS T833C/844ins68 SNPs influenced Hcy concentrations highlighting the importance of environmental factors; and gene–gene interactions allude to epistatic effects.

1. Introduction

Elevated homocysteine (Hcy) concentrations (hyperhomocysteinemia) have been implicated in the development of cardiovascular disease (CVD) (Humphrey et al., 2008) and various other conditions including cancer (Kohaar et al., 2010; Muntjewerff et al., 2006; Yang et al., 2012; Zintzaras, 2010). The prevalence of both CVD (Sliwa et al., 2008) and cancer (Ferlay et al., 2010) is increasing within African populations and is ascribed to urbanization in association with unhealthy behaviors (Vorster, 2002).

At the population level, the inherited genetic risk conveyed through Hcy towards the development of these diseases can be determined by establishing the frequency and genotype interactions between single nucleotide polymorphisms (SNPs) within genes encoding for enzymes involved in Hcy metabolism. The methylenetetrahydrofolate reductase (MTHFR) C677T (rs1801133), methionine synthase (MTR) A2756G (rs1805087) and cystathionine β-synthase (CBS) T833C (rs5742905)/844ins68 genetic alterations have been widely studied in Caucasian populations, but have rarely been reported separately or simultaneously in an African population. Since Africa is one of the most genetically diverse regions of the world (Schuster et al., 2010), studying these genetic alterations in Africans should prove especially informative. The value of investigating this unique population is not only in its genetic diversity, but also in its diversity in lifestyle, which is reported to strongly influence Hcy concentrations (de Bree et al., 2001).

The present study was undertaken to describe the frequencies of the previously mentioned SNPs and a novel SNP, *i.e.* CBS G9276A, in urban and rural black South Africans and to investigate their respective and

Abbreviations: CBS, cystathione-β-synthase; CVD, cardiovascular disease; CV, coefficient of variance; GGT, gamma (γ)-glutamyl transferase; Hcy, homocysteine; hs-CRP, high sensitivity C-reactive protein; HWE, Hardy–Weinberg equilibrium; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; PURE, *P*rospective *U*rban and *R*ural *E*pidemiology study; QFFQ, Quantitative food frequency questionnaire; RFLP, restriction fragment length polymorphism; SNPs, single nucleotide polymorphisms; THUSA, Transition and Health during Urbanization of South Africans study.

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collective influences on Hcy concentrations. This work will help us to understand the genetic and environmental determinants of tHcy concentrations in such a population with a view to informing disease prevention strategies on its behalf.

2. Materials and methods

2.1. Study design, population selection and ethics

This cross-sectional study was conducted on the baseline data of the South African leg of the *P*rospective *U*rban and *R*ural *E*pidemiology (PURE) study (Teo et al., 2009). Apparently healthy black South African volunteers of Tswana ancestry were recruited from towns and rural villages. Power calculations using results obtained from the Transition and Health during Urbanization of South Africans (THUSA) study indicated that 2000 participants were required. Individuals with reported chronic diseases or who used chronic medication were not eligible. Further details on the study sampling are presented elsewhere (de Lange et al., 2012).

Ethical approval was granted by the Ethics Committee of North-West University (Potchefstroom Campus) according to the principles outlined in the Declaration of Helsinki (Ethics number: 04M10). Written informed consent was required from subjects before their participation.

2.2. Blood sampling and storage

Nurses collected fasting venous blood samples and mixed the tubes by inversion before storing tubes on ice until centrifugation. Samples were centrifuged within 30 min of collection and the aliquoted serum, plasma and buffy coat were stored at −70 °C until analysis. Details on the preparation of aliquots are reported elsewhere (Nienaber-Rousseau et al., 2013).

2.3. Biochemical markers

For the analyses of gamma (γ)-glutamyl transferase (GGT) concentrations, a Sequential Multiple Analyzer Computer (SMAC) analysis was undertaken, using the Konelab™ auto analyzer (Thermo Fisher Scientific Oy, Vantaa, Finland). The SMAC method was also used to analyze serum high sensitivity C-reactive protein (hs-CRP) and serum creatinine. For all assays the coefficient of variance (CV) was <10%.

2.4. Determination of homocysteine concentrations

Total Hcy concentrations were determined from EDTA-treated plasma on the Abbott automated immunoassay analyzer (AxSYM). The determination of Hcy concentration was based on fluorescence polarization immunoassay technology (CV = 4.52%).

2.5. Deoxyribonucleic acid isolation and genotyping

QIAGEN FlexiGene DNA extraction kits were used to extract gDNA from citrate treated buffy coat. The absorbance ratio (260 to 280 nm) and yield of the extracted sample DNA were determined with the Nano-Drop spectrophotometer (ND-1000). Polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis was used to determine the genotypes. The PCR mixtures for the genotypes contained 0.125 nmol of each primer (the primers used are listed in Supplementary Table 1), 37.5 nmol $MgCl_2$, 0.5 nmol of each of the 2′-deoxynucleotide-5′-triphosphate (dNTPs), 0.625 U GoTaq® DNA polymerase and 50 ng DNA to a final volume of 25 μL. The PCR amplification was carried out in an iCycler thermal cycler (Bio-Rad, 582BR017217).

For genotype determinations, each batch of sample DNA was analyzed in parallel with the appropriate positive and negative controls to avoid misinterpretation from any lack of digestion or presence of contamination. To determine the MTHFR and MTR genotypes the fragments of the restriction enzyme digested PCR products were analyzed *via* electrophoresis and visualization of an ethidium bromide-stained 2% agarose gel. For the three CBS alterations both the PCR products and the digested PCR products were loaded on the gel. In order to ensure the validity of the results, the MTHFR, CBS and MTR loci investigated in this study were sequenced in 30 randomly selected subjects from the PURE study and these results were compared to those obtain through the PCR-RFLP.

Two researchers, blind to the Hcy phenotype, scored the genotypes independently and merged the electronic spreadsheets with the outcomes. Discrepancies in interpretation were resolved by repeating the PCR-based RFLP analysis. Due to monetary constraints these samples could not be sequenced in addition to the 30 randomly selected samples and were excluded from the analysis.

2.6. Anthropometrical assessment

Anthropometrists accredited by the International Society for the Advancement of Kinanthropometry (ISAK) measured body mass with an electronic scale (Precision Health Scale, A&D Company, Tokyo, Japan) and height with a stadiometer (IP 1465, Invicta, London, UK).

2.7. Questionnaires

Demographic, health and lifestyle information was obtained from an interviewer-based questionnaire developed for the PURE study, but adapted and standardized for use in South Africa. In addition, food and beverage consumption was assessed with a validated quantitative food frequency questionnaire (QFFQ) developed in South Africa (MacIntyre et al., 2001). The dietary data was computerized using *FoodFinder3*® and sent to the Medical Research Council of South Africa for verification and nutrient analyses.

2.8. Statistical analysis

Statistical analyses were conducted using the software package *Statistica*® (Statsoft Inc., Tulsa, Oklahoma, USA) and the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). The HIV-positive subjects were asymptomatic and did not use antiretrovirals and so were not excluded from the analysis. Data was tested for normality and was normalized through logarithmical transformation where necessary.

Pearson correlations were calculated to determine the linear associations between Hcy concentrations and other variables and to identify possible confounders. Descriptive statistics for the group as a whole and for subjects subdivided according to gender and area of residence were calculated.

Independent t-tests (for continuous data) and cross-tabulation (for categorical data) were performed to determine whether there were significant differences in measured variables between population subdivisions.

Concordance of the observed genotype frequencies with those expected according to the assumptions of Hardy–Weinberg equilibrium (HWE) was tested for using Pearson chi-squared analysis (Hardy, 1908). Analyses of covariances (ANCOVA) were conducted to determine the effect of the genotypes on the Hcy phenotype.

Potential joint influences between gender, residential area and genotypes including gene–gene interactions in relation to Hcy concentrations were determined through one-, two- and three-way ANCOVA. The Tukey honest significant difference for unequal *n* and Bonferroni *post hoc* tests were used to adjust for multiple comparisons and to establish between which groups the specific significant differences occurred.