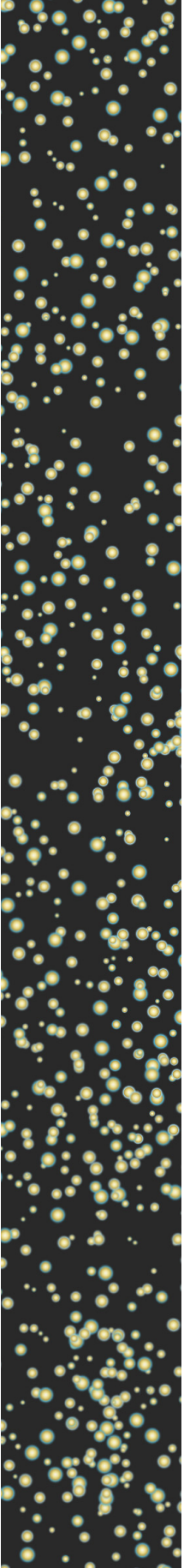
Exploring Redox Biology in physiology and disease

Koning, Anne



Chapter 6

A CBS gene variant in kidney transplant patients might positively affect graft survival

Anne M. Koning
Henri G.D. Leuvenink
Ilja M. Nolte
Harry van Goor

Chapter

Abstract

Background

The *CBS* gene regulates the expression of cystathionine beta-synthase (CBS), a key enzyme in the production of endogenous hydrogen sulfide (H_2S). Ischemia/reperfusion of the kidneys impairs CBS activity and consequently H_2S production. Partial restoration of CBS activity causes an increase of the H_2S level and reduction of ischemia/reperfusion damage. We hypothesize that variations in the expression of H_2S producing enzymes caused by single nucleotide polymorphisms (SNPs) affect susceptibility of kidney grafts to ischemic damage, and consequently affect graft survival.

Methods

The genotype of 1271 donor and recipient pairs were determined for seven tag SNPs in the *CBS* locus. These SNPs were analyzed for association with primary non-function (PNF), delayed graft function (DGF), first years biopsy proven acute rejection (AR), death-censored graft survival and patient survival.

Results

Univariable analysis showed that graft survival is improved in kidney transplant recipients that homozygously carry the minor allele of rs11203172. Furthermore, no PNF was seen in these patients. Multivariable analyses showed no significant associations, most probably due to the relatively low number of patients in this group and the number of parameters studied.

Conclusions

Kidney transplant patients who homozygously carry the minor allele of the *CBS* gene SNP rs11203172 seem to have a better graft survival. Since CBS is markedly reduced under ischemic conditions we assume that this finding is of considerable importance. Therefore, we plan to further study this *CBS* gene variant and unravel the role of the *CBS* gene in renal protection.

1 Introduction

The *CBS* gene regulates the expression of cystathionine beta-synthase (CBS). Together with cystathionine γ-lyase (CSE) this enzyme is responsible for the majority of the endogenous production of hydrogen sulfide (H_2S) through breakdown of L-cysteine.(1,2) H_2S is now recognized as a gaseous transmitter that can protect the body from ischemic insults.(2,7) Even though CBS predominantly produces H_2S in the nervous system, this enzyme is also highly expressed in the kidneys, where H_2S plays a role in the regulation of kidney function via both vascular and tubular actions.(2,8) Ischemia/reperfusion of the kidneys has been shown to impair CBS activity and consequently H_2S production.(9,10) Partial restoration of CBS activity was shown to not only increase the H_2S level but also reduce ischemia/reperfusion induced lipid peroxidation and cell damage in the kidney tissue.(10)

CBS knockout mice which were generated to study this gene in relation to homocystinuria usually die within 5 weeks of birth.(11) Surprisingly, introduction of a human variant of the *CBS* gene in these mice entirely prevents this neonatal mortality.(12) At least 150 mutations in the *CBS* gene have been found to cause CBS deficiency. As a result of CBS deficiency hypermethioninemia occurs by excessive remethylation of homocysteine, the primary metabolite that is abnormally accumulated.(13)

We hypothesized that variations in the expression of H_2S producing enzymes caused by single nucleotide polymorphisms affect the susceptibility of kidney grafts to ischemic damage, and consequently affect graft survival.

To test this hypothesis we genotyped kidney transplant recipients and donors for seven selected SNPs in the *CBS* locus. After the transplantation the recipients were followed up and primary graft nonfunction, delayed graft function, first years biopsy proven acute rejection, death-censored graft survival, patient survival and several other clinical parameters were documented.

2 Materials and Methods

2.1 Study population

Between March 7, 1993 and February 12, 2008, 1430 kidney transplants took place in our center. From these we selected 1271 donor and recipient pairs. Cases of three or more kidney transplants, simultaneous transplantation of other organs (pancreas, liver, lung and intestine) and technical problems during the procedure were excluded. The genotype of one donor and recipient pair could not be determined and four patients were lost to follow-up. Informed consent was given by all patients. Donor, recipient and transplant characteristics and transplant outcome were documented as shown in Table 1 (next page).

Table 1: Donor, recipient and transplant characteristics according to the recipients rs11203172 genotype (homozygous for the minor allele (TT) compared to heterozygous (TG) or homozygous for the major allele (GG))

Variable	**TT** (n = 40)	**TG/GG** (n = 1231)	**P value**[*]
Donor characteristics			
Age[†]	46 (12 - 63)	47 (7 – 74)	0.54
Gender (% female)	53	49	0.68
Donor type (%):			
Living	25	22	0.02
Brain dead	75	62	
Non heart beating	0	16	
Recipient characteristics			
Age[†]	55 (17 - 67)	49 (7 – 74)	0.31
Gender (% female)	33	42	0.22
PRA level > 5%	7	264	0.17
Previous transplants (% second)	5	9	0.38
Primary kidney disease (%):			
Glomerulonephritis	7	161	0.50
Adult polycystic disease	6	161	
Renal vascular disease	7	138	
IgA nephropathy	4	94	
Pyelonephritis	3	145	
Diabetes	2	49	
Chronic, unknown	4	164	
Other	5	265	
No data	2	54	
Initial immunosuppression (%):			
Corticosteroids	98	94	0.40
Mycophenolic acid	68	72	0.59
Cyclosporin	78	86	0.15
Azathioprine	5	6	0.85
Tacrolimus	18	7	0.02
ATG	13	8	0.30
Anti-CD3 moab	5	1	0.63
Interleukin-2 RA	10	16	0.32
Sirolimus	5	3	0.45
Transplant characteristics			
First warm ischemia time[†]	0 (0 – 5)	0 (0 - 55)	0.02
Cold ischemia time[†]	19 (2 - 37)	19 (1 – 41)	0.37
HLA mismatches (% of 0 mismatches)	30	19	0.33
Transplant outcome			
Primary graft nonfunction (%)	0	5	0.15
Delayed graft function (%)	23	33	0.16
Biopsy proven acute rejection (first year) no (%)	35	33	0.59
Death censored graft survival	0.124 (0.017 – 0.882)[§]	1	0.01[‡]
Patient survival	1.246 (0.679 – 2.287)[§]	1	0.48[‡]

[*]*All P values are two-sided. Mann-Whitney test for continuous variables, and Chi-square test for binary variables.*
[†]*Median (range).*
[‡]*Log rank test.*
[§]*Hazard ratios (95% confidence interval)*
Abbreviations: PRA; panel reactive antibody, HLA; human leukocyte antigen, ATG; antithymotic globulin, moab; monoclonal antibody, RA; receptor antagonist

2.2 DNA isolation and genotyping

DNA was extracted from peripheral whole blood in recipients and living donors and from lymph nodes or spleen lymphocytes in deceased donors using a commercial kit following the manufacturer's instructions. The DNA was transferred into 2 ml Eppendorf tubes and stored at -20 °C. Absorbance at 260nm was measured using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies) and the DNA concentration was calculated by the NanoDrop nucleic acid application module. As a measure of DNA purity 260/280 and 260/230 absorbance ratios were assessed. Where samples failed to meet the minimum DNA concentration and purity recommended for Illumina genotyping, repeated isolation attempts were made.

Donors and recipients were genotyped for seven SNPs in the *CBS* locus: rs234706, rs234713, rs1788484, rs1789953, rs2851391, rs11203172, rs12329764. Using Haploview software, we selected these SNPs as *CBS* tag SNPs based on linkage disequilibrium patterns in the combination of two populations from the HapMap project, CEU (Utah residents with Northern of Western European ancestry) and TSI (Tuscans in Italy), which together consist of 205 individuals.(14)

Genotyping of the selected SNPs was performed using the Illumina VeraCode GoldenGate assay kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. Genotype clustering and calling were performed using BeadStudio Software (Illumina).

2.3 Study end-points

The primary study end points were primary graft non-function (PNF, defined as non-functioning of the allograft from transplantation onwards), delayed graft function (DGF, defined as the requirement of dialysis within the first week after transplantation), biopsy proven acute rejection (according to Banff classification) during the first year after transplantation (AR), death censored graft survival (defined as the absence of the need for dialysis or re-transplantation), and patient survival.

2.4 Statistical analysis

The statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 18.0. To assess the association between the SNPs and outcome the three genotypic groups were compared using Kruskal-Wallis tests for continuous variables and Chi-square tests for categorical variables. The Mann-Whitney U test was applied to compare continuous variables between two genotypic groups (homozygous carriers of a minor allele versus others). Kaplan-Meier survival curves and log rank tests were used for univariable analysis of the effect of SNPs on graft survival and patient survival. For the univariable associations that were found to be statistically significant ($P < 0.05$), multivariable Cox and logistic regression analyses were performed. Multivariable analyses were performed with covariates that are known from literature to influence graft outcome. These include: donor age, recipient age, number of previous transplants of recipient, donor type, cold ischemia time, first warm ischemia time, percentage of panel reactive antibodies and the number of human leukocyte antigen mismatches and primary recipient kidney disease. For all analyses a Bonferroni correction for

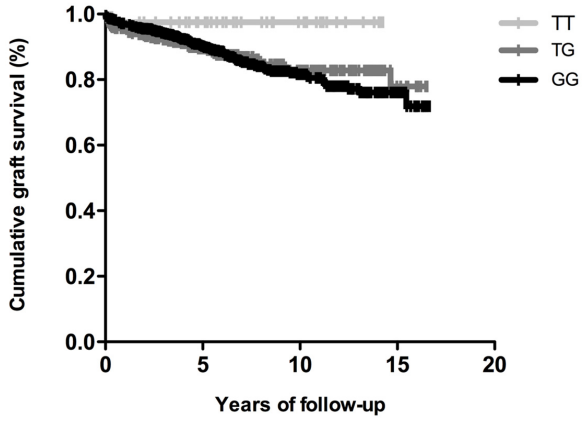
multiple testing was applied. Since we tested seven SNPs a P value < 0.007 was considered to be statistically significant.

3 Results

Univariable analysis showed improved graft survival in kidney transplant recipients that homozygously carry the minor allele (T) of rs11203172 (P = 0.03 (three genotypic groups); P = 0.01(homozygous carriers of the minor allele versus others), Fig. 1). The risk of graft failure was over 8 times lower for homozygotes for the minor allele compared to others. Only 1 in 40 (2.5%) of these recipients experienced graft failure, compared to 70 in 374 (18.7%) of heterozygous recipients and 144 in 857 (16.8%) recipients homozygous for the major allele (G). Furthermore, no PNF was seen in the 40 recipients that homozygously carry the minor allele (P = 0.027 (three genotypic groups); P = NS when comparing with both other groups together). There were no significant differences in baseline characteristics between recipients that homozygously carry the minor allele and others except for donor type, Tacrolimus treatment, and first warm ischemia time. Tacrolimus treatment and first warm ischemia time in turn were not associated with death-censored graft survival. The association between rs11203172 and graft survival remains nominally significant after correction for donor type (P = 0.04). After Bonferroni correction for multiple testing the associations between rs11203172 and graft survival and PNF were not significant. Also, multivariable analyses with all covariates mentioned above showed no significant associations.

In recipients the minor allele frequency (MAF) of rs11203172 was 17.6% and it was 17.8% in donors, but the rs11203172 genotypes of donors were not associated with graft survival or PNF in recipients, nor were any of the other SNPs studied here. Also, no associations were found in either donors or recipients between the SNPs and DGF, AR, or patient survival.

Figure 1: Kidney graft survival by recipient rs11203172 genotype, censored for death with a functional graft



TT = homozygous for the minor allele (T), TG = heterozygous, GG = homozygous for the major allele (G).

4 Discussion

The major finding of this study is the association between the *CBS* gene SNP rs11203172 and graft survival in univariable analysis. From previous work it is known that the *CBS* mRNA is markedly reduced after renal ischemia.(9,10) We assume that our finding is of considerable importance regarding the pathogenesis of kidney transplant failure.

Univariable analysis showed a nominally significant association between rs11203172 and graft survival in kidney transplant recipients. Although this association disappeared after adjustment for possible confounders in a multivariable analysis, this is most likely due to the relatively low number of patients that homozygously carry the minor allele. Therefore, we believe that this finding is of considerable importance as graft failure occurred in only one of the recipients homozygous for the minor allele. Further research, for instance by studying this SNP in another cohort, is needed to elucidate this association.

Since rs11203172 is located on an intron, the function of the *CBS* gene is not directly affected by the genotypic variations of this SNP. However, SNPs in noncoding regions may affect the expression level of genes. Furthermore, non-functional SNPs are often in linkage disequilibrium with SNPs that are located in a functional region and can therefore act as useful genetic markers. Because of the strong association between rs11203172 and graft survival shown in this study, we believe this to be likely in this case. Analyzing both donor and recipient genotypes enables differentiation between local, intra-renal and systemic, extra-renal influences. Since no association was found between donor rs11203172 and graft survival we expect related functional SNPs to have systemic effects.

CBS is known to be one of the key enzymes responsible for production of endogenous H_2S.(1,2) Measuring H_2S requires pretreatment of the plasma with a zinc solution. Since a historical database was used for this study, data on H_2S values were not available. However, previous studies showed that H_2S produced by CBS is anti-inflammatory, regulates blood pressure and scavenges reactive oxygen species (ROS).(2-4,7) Furthermore, H_2S may be able to induce hypometabolism in cells, thereby protecting them against ischemic insults.(5,6) Since the process of kidney transplantation is associated with inflammation, ischemia and production of ROS, we consider CBS to be a key enzyme in the protection against graft failure. Increasing the renal content of H_2S, either through exogenous administration or through stimulation of the endogenous production by stimulation of CBS, may be a promising strategy to minimize ischemia/reperfusion injury during renal transplantation.

As described above, the investigated tag SNPs were chosen based on the combination of two populations from the HapMap project.(14) Naturally, several differences may exist between these populations and the population of the present study, in particular the kidney transplant patients. For this reason, one could question how well tag SNPs defined in one population perform in another. However, research has shown that tag SNPs are often highly portable across human populations.(15) Nevertheless, a high portability as a mean does not assure portability for each tag SNP and because of that, it is possible that we missed a SNP in the *CBS* locus that is also graft outcome-associated.

Using Haploview software, we specifically determined seven tag SNPs in this gene that would enable us to subsequently predict the majority of all common genetic variations by

haplotype reconstruction, thereby reducing the number of tests. Even so, we analyzed seven SNPs and therefore correction for multiple testing was indicated. The association we found between rs11203172 and graft survival disappeared after Bonferroni correction. However, as mentioned before, this is most likely due to a lack of power. Replication in another, independent cohort is needed to confirm this association.

Taken together, we found a SNP in the *CBS* gene that is significantly associated with graft survival. Functional SNPs in the close vicinity may cause variations in H_2S during the kidney transplant process. To elucidate the exact mechanisms by which CBS can influence graft survival, further research is necessary.

Funding

This project has been supported by the Foundation: "De Drie Lichten" in The Netherlands.

Conflicts of interest

None declared.

References

1. Singh S, Padovani D, Leslie RA, Chiku T, Banerjee R. Relative contributions of cystathionine beta-synthase and gamma-cystathionase to H_2S biogenesis via alternative trans-sulfuration reactions. *J Biol Chem* 284(33): 22457-22466, 2009
2. Xia A, Chen L, Muh RW, Li P, Li N. Production and actions of hydrogen sulfide, a novel gaseous bioactive substance, in the kidneys. *JPET* 329: 1056–1062, 2009
3. Wang R. Two's company, three's a crowd: can H2S be the third endogenous gaseous transmitter? *FASEB J* 16: 1792-1798, 2002
4. Boehning D, Snyder SH. Novel neural modulators. *Annu Rev Neurosci* 26: 105-131, 2003
5. Blackstone E, Roth MB. Suspended animation-like state protects mice from lethal hypoxia. *Shock* 27: 370-372, 2007
6. Bos EM, Leuvenink HGD, Snijder PM, Kloosterhuis NJ, Hillebrands J, Leemans JC, Florquin S, Van Goor H. Hydrogen sylfide-induced hypometabolism prevents renal ischemia/reperfusion injury. *J Am Soc Nephrol* 20: 1901-1905, 2009
7. Szabo C. Hydrogen sulfide and its therapeutic potential. *Nat Rev Drug Discov* 6(11): 917-935, 2007
8. Stipanuk MH, Beck PW. Characterization of the enzymatic capacity for cysteine desulphhydration in the liver and kidney of the rat. *Biochem J* 206(2): 267-277, 1982
9. Wu N, Siow YL, O K. Ischemia/reperfusion reduces transcription factor Sp1-mediated cystathionine beta-synthase expression in the kidney. *J Biol Chem* 285(24): 18225-18233, 2010
10. Xu Z, Prathapasinghe G, Wu N, Hwang S, Siow YL. Ischemia-reperfusion reduces cystathione beta-synthase-mediated hydrogen sulfide generation in the generation in the kidney. *Am J Physiol Renal Physiol* 297: F27-F35, 2009
11. Watanabe M, Osada J, Aratani Y, Kluckman K, Reddick R, Malinow MR, Maeda N. Mice deficient in cystathionine beta-synthase: Animal models for mild and severe homocyst(e)inemia. *Proc Natl Acad Sci USA* 92: 1585-1589, 1995
12. Wang L, Chen X, Tang B, Hua X, Klein-Szanto A, Kruger WD. Expression of mutant human cystathionine beta-synthase rescues neonatal lethality but not homocystinuria in a mouse model. *Hum Mol Genet* 14: 2201-2208, 2005
13. Mudd SH. Hypermethioninemias of genetic and non-genetic origin: a review. *Am J Med Genet Part C Semin Med Genet* 15: 3-32, 2011
14. The International HapMap Consortium. Integrating common and rare genetic variation in diverse human populations. *Nature* 467: 52-58, 2010
15. Gonzalez-Neira A, Ke X, Lao O, Calafell F, Navarro A, Comas A, Cann A, Bumpstead S, Ghori J, Hunt S, Deloukas P, Dunham I, Cardon LR, Bertranpetit J. The portability of tagSNPs across populations: a worldwide survey. *Genome Res* 16: 323-330, 2006