 © F. K. Schattauer Verlagsgesellschaft mbH (Stuttgart) 74 (6) 1497–500 (1995)

An Amino Acid Polymorphism in Histidine-rich Glycoprotein (HRG) Explains 59% of the Variance in Plasma HRG Levels

B. C. Hennis, D. I. Boomsma[1], P. A. van Boheemen, L. Engesser, P. Kievit, G. Dooijewaard, C. Kluft

From the Gaubius Laboratory TNO-PG, Leiden, The Netherlands, [1]Department of Psychonomics, Free University, Amsterdam, The Netherlands

Summary

A pedigree-based maximum likelihood method developed by Lange et al. (12) was used to study the contribution of a newly defined di-allelic polymorphism in histidine-rich glycoprotein (HRG) to the plasma levels of HRG. In four families (n = 99) and 20 volunteers we found a heritability of 70%, an age effect of 3% and an effect of individual environmental factors of 27%. These results are remarkably similar to the results found in a previous parent-twin study in which a heritability of 69% and an effect of random environment of 31% was found. The overall genetic influence in the present study can be subdivided into an effect of 59% by the HRG phenotype and 11% by residual genetic factors. The influence of the HRG phenotype of 59% can entirely be explained by adding up the effect of the two alleles that make up the phenotype. These results indicate a codominant inheritance pattern of HRG levels in which the genetic influence can almost completely be ascribed to the additive effect of the di-allelic HRG locus whereas only a small part is due to other loci.

Introduction

The human plasma component histidine-rich glycoprotein (HRG) is a single chain protein which is thought to act as a modulator of coagulation and fibrinolysis (1). In a few studies a slightly higher prevalence of elevated HRG plasma levels has been found in groups of patients with venous thrombosis (2, 3, 4). In addition, inherited elevation of HRG has been suggested in several thrombophilic families (5, 6, 7). However, it is as yet not clear whether the apparent relationship between elevated HRG levels and thrombosis is causal or coincidental.

Recently, we described a family in which an association was observed between elevated HRG levels and a specific allele of a dinucleotide repeat polymorphism (8). The polymorphism is located between the last two exons of the structural gene for HRG which encloses about 10kb and has lately been localized on chromosome 3q28-q29 (9). The observed association may indicate that a variant of the HRG gene which is coupled to this specific allele of the di-nucleotide repeat polymorphism is responsible for the high HRG levels in this family. However, it is not known whether the gene causing elevated HRG levels is unique to this family or whether it is also present in the population.

Correspondence to: Dr. B. C. Hennis, Gaubius Laboratory, TNO-PG, P.O. Box 2215, 2301 CE Leiden, The Netherlands – FAX Number: +31 71 5181904

The presumed genetic influence on HRG levels was also confirmed in a parent-twin study (10). In this study evidence was found for a simple additive genetic model of inheritance in which 69% of the variance in HRG levels could be accounted for by genetic factors. The residual 31% of inter-individual variance was due to non-genetic factors unique to each individual. No evidence was found for the influence of common environment shared by family members. We then suggested that the substantial heritability justifies the search for quantitative trait loci. This is possible if suitable markers are available for the HRG gene or for loci that may influence HRG levels.

Up to now no suitable marker for the HRG locus was available. However, recently we described two common molecular weight variants of HRG (11). The two forms noted as form 1 (77 kD) and form 2 (75 kD) appear as a doublet on SDS-PAGE and have frequencies of 0.35 and 0.65, respectively. The three possible phenotypes (homozygous: 1-1 or 2-2 and heterozygous: 1-2) followed a mendelian inheritance pattern indicating that the two forms are encoded by different HRG alleles. With the availability of this polymorphism (i.e. the phenotype) for HRG it is now possible to discriminate beween influences of different alleles at the HRG locus itself and contributions of alleles at other loci. In the present study we used a pedigree-based maximum likelihood method developed by Lange et al. (12) to study the contribution of the HRG locus to the variance in plasma HRG levels in four families (n = 99) supplemented with 20 healthy volunteers.

Subjects and Methods

Selection of Probands

This study was approved by the Medical Ethical Committee of HGO-TNO and informed consent was obtained from family members and volunteers. The probands and their families used in this study were selected from a previous study on the relationship between plasma HRG levels and idiopathic thrombophilia (3). Probands with thromboembolic disease, a persistently elevated HRG level (≥145%) and a family history of thrombosis were included when at least two other family members had had thromboembolic disease. Four families enclosing 99 individuals in three generations were included in the present study. One of the four families (J.K.) has partly been described by Engesser et al. (5).

A history of thrombosis is found in 16 family members including the probands, but no relationship is found between HRG levels and thrombosis in these families (Chi-squared difference test (1) = 1.04).

Age, gender and HRG levels of volunteers (n = 20) were previously described in a study on the longitudinal variation of HRG (13).

Blood Collection

Blood was obtained from family members and volunteers by a single venipuncture using vacutainer tubes (Becton Dickinson, France) with either sodium citrate or sodium EDTA as anticoagulant. Samples were placed on ice and centrifuged immediately at 3000 g for 30 min at 4 °C. Plasma was separated and stored at –80 °C until use. Samples were thawed only once immediately before measurement.

HRG Measurement

Plasma HRG levels were measured by radial immuno diffusion (14) as described previously (10). HRG concentrations were calculated from a standard dilution series of pooled plasma obtained from 26 healthy volunteers. The same anticoagulant was not in all families used to prepare plasma. In family W.K./C.B. and in the volunteers sodium citrate was used, in family F.Z., J.K. and B.Z. sodium EDTA was used. To circumvent differences in measurement due to variation in anticoagulant, calibration lines were prepared both from EDTA and citrated pooled plasma. HRG levels were expressed as a percentage of pooled plasma taking citrated pooled plasma as 100%. The inter-assay coefficient of variation of the duplicate measurements was 10%. Using this method HRG levels were determined in 126 healthy individuals. The mean HRG level was 99% (SD = 20%) and the range was 56%-145%. Plasma levels of family members were determined only once since individual HRG levels tend to be very stable in time (13).

Detection of HRG Phenotypes

HRG was isolated using a small-scale batch-wise isolation procedure as described previously (11). Individual HRG phenotypes were determined by immunoblotting of the purified HRG.

Statistics

For statistical analysis we used a pedigree-based maximum likelihood method developed by Lange et al. (12), in which for a given pedigree of n individuals a vector of observations (x) is defined and a vector of expected values (E(x)), that can depend on measured variables such as sex, age or measured phenotype. The covariances between the residual part of the observations, i.e. the part that is not accounted for by the measured phenotype or other variables, depend on the relationships between the pedigree members and on the genetic model assumed for the observations. Throughout we have modelled the variance in HRG not accounted for by the measured phenotype as consisting of additive genetic and random environmental variance, since we found no evidence for the influence of common environment in a parent-twin study (10). For a given E(x) and expected covariance matrix Σ, the log-likelihood of obtaining the observation vector x is:

$$L = -{}^1/_2 \ln|\Sigma| - {}^1/_2 (x\text{-}E(x))' \Sigma^{-1} (x\text{-}E(x)) + \text{constant};$$

where $|\ |$ denotes matrix determinant and ' denotes matrix transpose.

The joint log-likelihood of obtaining all pedigrees is the sum of the log-likelihood of the separate pedigrees. Estimation of parameters involves selection of parameter values under a specific model which maximizes the joint likelihood of all pedigrees. The likelihoods obtained for different models can be compared with chi-squared difference tests where x2 = 2 (L1-L0) and L1 and L0 denote the log-likelihood for the general (H1) and the constrained (H0) hypothesis. The degrees of freedom (df) for this test are equal to the number of independent parameters be-tween H1 and H0 (12). The FISHER package (17) was used for genetic modeling. Ascertainment correction was carried out by conditioning on the probands. The effects of the measured phenotype were first considered by estimating 3 means for the 3 genotypes (2 homozygotes and 1 heterozygote) and alternatively by estimating a mean effect for each of the two alleles and summing the allelic effects. The difference in likelihood (with 1 df) between these two models provides a test of interaction between 2 alleles at the HRG locus (i.e. genetic dominance).

Results

HRG Phenotypes

The two forms of HRG (1 and 2) that make up the phenotype followed a mendelian pattern of inheritance in all four families. The inheritance pattern strongly suggests that the two protein variants of HRG are encoded by two different alleles of the HRG locus. This is recently confirmed by the elucidation of the underlying molecular defect. The difference in molecular weight is due to a di-allelic amino acid polymorphism in the second cystatin domain of HRG (11).

Phenotype and HRG Level

Descriptive statistics of mean HRG levels and mean ages for each phenotype are presented in Table 1. The highest mean levels of HRG were found in individuals homozygous for form 1 and the lowest levels in homozygotes for form 2, whereas heterozygotes had an intermediate HRG level. Frequencies of form 1 and form 2 in this data set were 0.42 and 0.58, respectively. Statistical modelling of the influence of HRG

Table 1 Descriptive statistics of mean HRG levels and mean ages in individuals with the same phenotype (excluding the probands)

Phenotype	Number of individuals	Mean HRG level (%)	Mean Age
1-1	20	156	46
1-2	55	121	37
2-2	39	93	40
All	114	118	40

Table 2 Results of maximum likelihood analysis of HRG levels in four families and 20 volunteers. Log-likelihood estimates for 6 models are shown

Model	Log-likelihood	Tested against model	Chi-squared difference test	df for difference test
I	–383.02			
II	-383.04	I	0.02	1
III	–388.36	II	10.65*	1
IV	–431.88	II	97.68*	2
V	–389.36	II	12.65*	1
VI	–383.35	II	0.63*	1

* significant decrease in likelihood indicating that the effect tested in this model is significant.

Model definition:

I. Most general model allowing for: (i) effect of gender, (ii) effect of age, (iii) differences in mean values of HRG phenotypes, (iv) additive genetic influence and (v) random environmental variability.
II. No gender difference.
III. No age regression.
IV. Means of all phenotypes are the same.
V. No residual genetic variance.
VI. Effect on phenotypic mean is sum of effect of each allele.

Model VI is the best model in this analysis with significant contributions of the factors tested in model III, IV and V. This indicates that there are: (i) no effects of gender, (ii) significant effects of age, (iii) significant differences in mean levels of different phenotypes, (iv) significant effects of residual additive genetic factors (i.e. the contribution of other genes than HRG) and (v) that the effect of the two alleles on the phenotypic mean is additive.

Table 3 Parameter estimates for model II and VI. Mean levels (in percentage pooled plasma) and standard errors are given for HRG phenotypes and alleles in the different models. Parameter estimates and standard errors are given for the age regression and the variability due to genetic factors other than HRG and environmental factors

	Model II Mean (s.e.m.)		Model VI Mean (s.e.m.)
Phenotype 1-1	140.9 (6.09)	Allele 1	69.4 (2.74)
Phenotype 1-2	106.6 (4.56)	Allele 2	38.1 (2.30)
Phenotype 2-2	77.6 (4.89)		–
	Parameter estimate (s.e.m.)		Parameter estimate (s.e.m.)
Age regression	0.32 (0.09)		0.33 (0.09)
Genetic variance	100.7 (47.0)		95.7 (44.7)
Environmental variance	218.7 (45.0)		224.1 (44.4)

phenotypes on the HRG plasma level was done by considering the phenotypes of individuals of four different families and 20 volunteers. In a strict sense the families were only four independent observations. To take this into account we used a pedigree-based maximum likelihood method developed by Lange et al. (17). The statistical analysis of the HRG level is shown in Table 2 as log-likelihood estimates for 6 models (as indicated in the legends of Table 2). The best model (VI) included contributions of age, alleles 1 and 2, residual genetic factors and random environmental factors.

Parameter estimates of the contributing factors from two models (II and VI) are shown in Table 3. Environmental factors explained 27% of the total variance and age explained 3%. Genetic factors explained 70% of the variance of which 59% could be ascribed to the phenotype and 11% to other genetic factors. In model VI the effect of the phenotype was considered by estimating a mean effect for each of the two alleles and summing the allelic effects. From this test we conclude that the two alleles at the HRG locus act completely additively.

Discussion

In the present study we used four families with 99 individuals and a sample of 20 volunteers, to determine the effect of the HRG locus on HRG plasma levels. The contribution of genetic and environmental factors to the variance in HRG levels has previously been established in a parent-twin study (10). At that time it was not possible to discriminate beween influences of different alleles at the HRG locus itself and contributions of alleles at other loci. However, with the newly defined di-allelic polymorphism of HRG (11), we have now been able to study the influence of the HRG locus on plasma HRG levels.

Using this polymorphism we found a heritability of 70%, an age effect of 3% and an effect of individual environmental factors of 27%. The results found in the parent-twin study and this study are remarkably similar given the two different approaches. In the parent-twin study an unselected sample of 160 Dutch families consisting of adolescent twins and their parents was used, whereas in this study HRG data from 4 pedigrees selected for familial thrombophilia and elevated HRG levels were employed. It has been suggested that twin studies provide consistently higher estimates of heritabilities than family studies. Our data very convincingly show that this has not always to be the case.

The overal genetic influence of 70% could be split up into an effect of 59% by the two forms of HRG and 11% by residual genetic factors. Two statistical models (II and VI, see Table 2) were used to estimate the variance in HRG levels explained by genetic factors. In model II the three possible phenotypes were used whereas in model VI the separate alleles which make up the phenotype were used. The latter model was not significantly different from model II and should therefore be considered as more favoured because one degree of freedom is gained (Table 2). Mean levels for each phenotype could be predicted by estimating a mean effect on the HRG level for each of the two alleles and summing the allelic effects (Table 3). From this we can deduce that the effect of the two alleles on the measured HRG level is completely additive. The inheritance patterns of elevated HRG levels observed in several families are therefore unlikely to be dominant as was suggested previously (5, 7, 18). Instead, the inheritance pattern of HRG levels has to be considered as codominant with a contribution of both alleles which can simply be added up. In addition, both alleles are common alleles and there is a residual variance of 38% (remaining genetic and environmental variance). It is therefore reasonable to expect a normal distribution instead of a bimodal distribution for HRG levels of individuals from the general population. Distributions of HRG indeed resemble a normal distribution although they are slightly skewed to the right (10, 18, 19).

Individual HRG levels were determined only once in this study. With respect to this the question can be raised whether HRG levels are stable in time. However, individual HRG levels tend to fluctuate very little in time. In a previous study by de Bart et al. (13), the individual variation in HRG levels was determined in 20 volunteers during a six months period. The intra-individual variance accounts for only 1% of the total variance of HRG levels in this period.

Up to now, possible pathophysiological functions of HRG have been studied by means of plasma levels of HRG. Hypothetical mechanisms explaining the relationship between HRG and for example thrombosis are based on elevated levels of HRG which are due to an increase in the number of HRG molecules. However, as yet we do not know whether the reason for the difference in contribution of the two HRG alleles to the measured plasma level, is due to such an increase in HRG molecules. Therefore, if deviating HRG levels become important with respect to disease, the phenotype should be determined but attention should also be drawn to individual environmental circumstances which contribute about 30% to the HRG level.

Acknowledgements

The work was supported by grant 89.004 of the Dutch Thrombosis Foundation and grant 88.042 of the Netherlands Heart Foundation.

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Received May 8, 1995 Accepted after resubmission August 17, 1995