Genotype and Plasma Concentration of Cystatin C in Patients With Coronary Heart Disease and Risk for Secondary Cardiovascular Events

Michael Loew, Michael M. Hoffmann, Wolfgang Koenig, Hermann Brenner, Dietrich Rothenbacher

Objective—Cysteine proteases and their inhibitors such as cystatin C are assumed to play an important role in the pathogenesis of atherosclerosis and coronary heart disease (CHD). The aim of the study was to investigate the impact of cystatin C polymorphisms on cystatin C plasma levels and on prognosis of patients with CHD.

Methods and Results—Four polymorphisms in the promoter and exon 1 of the cystatin C gene (−82GC, −5GA, +4AC, and +148AG) and cystatin C plasma levels were determined in a cohort of 1013 patients with manifest CHD and aged 30 to 70 years participating in an in-hospital rehabilitation program. Patients were followed-up for a mean of 33.5 months and a combined end point (fatal and nonfatal cardiovascular disease [CVD] events) was used as the outcome variable. The major haplotype −82G/−5G/+4A was associated with cystatin C plasma levels with persons homozygous for the major haplotype having the highest levels (P=0.01). However, the haplotype was not associated with fatal and nonfatal cardiovascular events during the 3-year follow-up.

Conclusions—The major haplotype −82G/−5G/+4A of the cystatin C gene determines plasma levels of cystatin C with homozygous persons having the highest plasma levels, but there was no association with secondary CVD events in this study. (Arterioscler Thromb Vasc Biol. 2005;25:1470-1474.)

Key Words: coronary heart disease • cystatin C • genotype • prognosis • prospective study

Coronary heart disease (CHD) is the leading cause of death in Western industrialized countries.1 A large number of risk factors such as hypertension, diabetes, smoking, overweight, hyperlipidemia, physical inactivity, and others have been established as determinants for the development of CHD. Beyond these environmental and behavioral variables, genetic factors have been implicated in the development and prognosis of CHD and it has been assumed that the disease process itself might be highly polygenic.2 Polymorphisms in genes involved in blood coagulation, in the regulation of blood pressure, and in the metabolism of lipoproteins especially have been suggested as possible genetic determinants of CHD. In recent years, enzymes involved in vascular remodeling have come into the focus of interest. Metalloproteinases and their respective inhibitors have been found to play an important role in vascular remodeling by degradation of extracellular matrix.3

The finding of local cystatin C deficiency in human atherosclerotic and aneurysmal aortic lesions suggests the significance of an imbalance between cysteine proteases and their most abundant inhibitor, cystatin C.4 Cystatin C is a 13-kDa protein consisting of 120 amino acids encoded by a 7.3-kb gene located on chromosome 20, and it is constitutively secreted a short time after synthesis by virtually all nucleated cells.5 Recently, physiologically relevant polymorphisms have been identified in the promoter region and the signal peptide of the cystatin C gene, and an association between genotype, plasma level, and cardiovascular outcomes has been described assuming an imbalance between cystatin C and proteases as cathepsin, promoting plaque rupture.6 However, the final importance of cystatin C polymorphisms in human vascular disease is still discussed controversially and the prognostic value in patients with CHD is unknown.

We determined 4 polymorphisms in the promoter and exon 1 of the cystatin C gene in a cohort of 1,013 patients with CHD, namely the −82 G/C, the −5G/A, the +4A/C, and the +148 G/A polymorphism and analyzed the association of these polymorphisms, respectively, the major haplotype −82G/−5G/+4A with plasma levels of cystatin C and with prognosis.

Materials and Methods

Design and Study Population

We conducted a prospective cohort study with a 3-year follow-up of patients with CHD (International Classification of Diseases, 9th

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Materials and Methods

Design and Study Population

We conducted a prospective cohort study with a 3-year follow-up of patients with CHD (International Classification of Diseases, 9th...
Revised codes 410 to 414. Patients aged 30 to 70 years who participated in a 3-week in-hospital rehabilitation program between January 1999 and May 2000 in 2 cooperating clinics (Schwanenland-Klinik, Isny and Klinik im Südpark, Bad Nauheim, Germany) were enrolled in the study. In Germany, all patients after acute coronary syndrome or coronary artery revascularization are offered a comprehensive in-hospital rehabilitation program after discharge from the acute care hospital. The aim of this 3-week program is the reduction of cardiovascular risk factors, improvement of health-related quality of life, and the preservation of the ability to work (the latter only if a subject was still at work at the onset of disease, otherwise the prevention of nursing care). This in-hospital rehabilitation program usually starts ∼3 weeks after the acute event or coronary artery revascularization. In the current study, only patients who were admitted within 3 months after the acute event and/or coronary intervention have been included.

All subjects gave written informed consent. The study was approved by the Ethics Committees of the Universities of Ulm and Heidelberg and of the Physicians’ chamber of the States of Baden-Wuerttemberg and Hessen (Germany).

Data Collection
At the beginning of the in-hospital rehabilitation program all subjects filled out a standardized questionnaire containing sociodemographic information and medical history. In addition, information was taken from the patients’ hospital charts. Blood samples were taken from the participants before discharge from the rehabilitation center and stored at −80°C until analysis. In all patients, active follow-up was conducted 1 and 3 years after discharge. Information was obtained from the patient using a mailed standardized questionnaire. Information regarding secondary cardiovascular events and treatment since discharge from the in-hospital rehabilitation clinic was obtained from the primary care physician also by means of a standardized questionnaire. If a subject had died during follow-up, the death certificate was obtained from the local Public Health Department and the main cause of death was coded according to the International Classification of Diseases (9th Revision). Secondary cardiovascular disease (CVD) events were defined either as the main cause of death (as stated in the death certificate), nonfatal myocardial infarction (MI), or ischemic cerebrovascular event (stroke or transient ischemic attack). All nonfatal secondary events were reported by the primary care physicians.

Laboratory Methods
Genotyping was performed as described by Eriksson et al. For genotyping of the −82 G/C and +4 A/C polymorphisms, a polymerase chain reaction (PCR) fragment amplified by the forward primer, 5′-GATGGATGG GGAAGGACAG, and the reverse primer, 5′-CAGATGTCGACACAGGAG, was used and cleaved with restriction enzyme SacII. The −5 G/A polymorphism was investigated using the same PCR fragment as mentioned and the restriction enzyme Ddel. Genotyping of the +148 G/A polymorphism was performed using a PCR fragment amplified by the forward primer, 5′-TCTATACGTCGACCACCGTCTCG, and the reverse primer, 5′-TGCTGCGTT GTGTGACTCGT, and subsequently digested with the enzyme SacII.

Cystatin C concentrations in plasma were measured by immunoephelometry on a Behring Nephelometer II (Dade-Behring, Marburg, Germany). Creatinine was measured by standard methods and creatinine clearance was calculated according to the Cockcroft-Gault formula. Amino-terminal (NT)-proB natriuretic peptide (NT-proBNP) was measured by means of a 1-step enzyme immunoassay based on electrochemiluminescence (Elecys, Roche Diagnostics, Mannheim, Germany). All laboratory measures were performed in blinded fashion. Interassay coefficient of variation for cystatin C was 3.81%.

Statistical Methods
First, the study population was described with respect to various sociodemographic and medical characteristics. Differences in plasma levels of cystatin C according to cystatin genotypes were analyzed by analysis of variance and analysis of covariance, the latter to adjust for age and gender. Normalized linkage disequilibrium coefficients (D’) were calculated according to Ott. The association between cystatin C haplotype and cystatin C plasma levels was further investigated by linear regression adjusted for the following potential confounders: age (years), gender, creatinine clearance (continuously), history of MI (yes/no), hospital site (Isny, Bad Nauheim), and NT-proBNP (continuously).

A Cox proportional hazards model was used to assess the association of cystatin C haplotype and risk of secondary CVD events after control for covariates. Beside a model adjusting for age and gender, the following potential confounders were considered simultaneously in multivariate analysis: age (years), gender, body mass index (kg/m²), creatinine clearance (continuously), smoking status (never, current, ex-smoker), duration of school education (<10 years, ≥10 years), family status (married, other), history of MI (yes, no), history of diabetes mellitus (yes, no), severity of CHD (number of affected vessels at baseline), high-density lipoprotein cholesterol (continuously), hospital site (Isny, Bad Nauheim), and NT-proBNP (continuously). Covariates were added to the model if they were significant predictors of a secondary event at an α-level of 0.1 (age, smoking status, high-density lipoprotein cholesterol, history of diabetes mellitus, duration of school education, family status, and NT-proBNP), or if considered as a potential confounder (gender, body mass index, history of MI, severity of CHD, hospital site, and creatinine clearance). All statistical procedures were performed with the SAS statistical software package (version 8.02; Cary, NC).

Results
Overall, 1206 patients with a diagnosis of CHD within the past 3 months had been included in the study at baseline. Three-year follow-up information was complete for 1033 patients (85.7%). Full information of cystatin C plasma level and cystatin C genotype was available in 1013 patients.

Table 1 shows the main characteristics of the study population. Of the 1013 patients with a diagnosis of CHD, 58.1% had MI, and 42.7% of patients had 3-vessel disease. The mean age of patients was 59 years, most of them (56.8%) were between 60 and 70 years old, and 85.0% were male. For most patients, body mass index was between 25 and 30 kg/m².

Table 2 shows the differences in cystatin C plasma levels according to genotype. There was a significant association between polymorphism −82GC, −5GA, and +4AC of the cystatin C gene and plasma levels of cystatin C. The highest plasma levels of cystatin C were seen in patients homozygous for the wild-type (1.12 mg/L for genotype −82GG, −5GG, and +4AA, respectively). Heterozygous patients had lower plasma levels and the lowest levels were seen in patients, who were homozygous for the respective variation (0.98 mg/L for 82GG, 0.92 for 5AA, and 0.97 for 4CC). The +148AG polymorphism showed an U-shaped association between genotype and cystatin C plasma level, because heterozygous patients showed lowest values. The −82GC, −5GA, and +4GA polymorphism were in linkage disequilibrium in our study population (pair-wise D’ = 0.88 [−82GC/−5GA], 1.00 [−82GC/+4AC]), 0.88 [−5GA/+4AC], respectively). The major haplotype of these 3 polymorphisms was −82G/−5G/+4A and showed a similar association with cystatin C plasma levels as the single polymorphisms. The 41 patients without the major haplotype had lower cystatin C plasma levels compared with patients heterozygous or homozygous for the major haplotype −82G/−5G/+4A (P = 0.01).
In this prospective cohort study including 1013 patients with CHD, we found an association between cystatin C genotype and cystatin C plasma levels. The plasma concentration of cystatin C was 12.4% higher among patients homozygous for the major haplotype $-82G/-5G/4A$ than among patients without this haplotype. Persons heterozygous for the major haplotype showed an intermediate phenotype (intermediate plasma levels). However, the haplotype alone was not associated with risk of secondary cardiovascular events during the 3-year follow-up after control for covariates.

Our findings are partly in line with the results of Eriksson et al who had described an association between genotype and plasma levels of cystatin C in a cohort of healthy men and in postinfarction patients. For the $-82GC$, $-5GA$, and the $+4AC$ polymorphism we could confirm the previously reported association in our large cohort of 1013 patients with CHD after carefully adjusting for cofactors. For the $+148AG$ polymorphism, however, we could not confirm the reported association.

We also did not find an association between genotype alone and prognosis of CHD. In a nested case control study within the Physicians Health Study, Albert et al found no association between cystatin C plasma levels and reported intermittent claudication or need for peripheral artery surgery. Eriksson et al had described a clear association between cystatin C genotype and plasma levels of cystatin C, respectively, between plasma levels and severity of CHD. In this study, low plasma levels of cystatin C had been correlated with a higher mean number of angiographically determined coronary artery stenoses but not with the mean percentage of stenosis or lumen diameter. In contrast to this finding, a recent study showed that increasing levels of cystatin C were associated with a higher risk of subsequent death in patients with non-ST–elevation acute coronary syndrome but not with risk of subsequent MI.

Furthermore, we had previously reported an association between plasma levels of cystatin C and poor prognosis in this study population. Only high levels of cystatin C (i.e., $>1.24$ mg/L) were associated with risk of fatal and nonfatal cardiovascular events during follow-up. In the present analysis, mean plasma levels for the genotype determining the highest plasma levels were considerably lower ($1.13$ mg/L). Therefore, it is plausible that a direct association between the genotype and prognosis of CHD could not be found in the present analysis and the absence of an association between genotype and secondary CVD events in this study may partly reflect the moderate variation of mean cystatin C levels between the various genotype groups. The discrepancy to the result of Eriksson et al who had found an association between cystatin genotype and angiographically determined coronary stenosis may arise from the fact that rupture of coronary atheroma with preserved lumen often triggers acute fatal thrombosis, and this rupture may be influenced by other factors than intermediate cystatin levels.

Our study suggests that the investigated polymorphisms in the cystatin C gene only partly determine cystatin C plasma levels and their possible consequences, leaving room for other environmental or genetic factors. In a recent article,
Knight et al described that various factors such as age, gender, weight, smoking, and C-reactive protein were independently associated with higher plasma cystatin C levels after adjusting for renal function. Furthermore, rather than cystatin C alone, an imbalance between cystatin C expression and other proteases, eg, cathepsin S and cathepsin K, which have been found to be increasingly expressed in atheroma, may be of importance for prognosis of CHD by promoting plaque rupture and thus triggering acute fatal thrombosis. Consideration of many genetic factors in different proteases and their specific combinations in future work may provide more insight into cause, clinical implications, and thus prognosis of CHD.

**Study Limitations**

The following limitations of our study should be considered. Despite the large sample size of patients with CHD (>50% with MI), fatal CVD events during follow-up were rare in our study population. This is explained by the fact that case fatality of MI is highest during the prehospital and early hospital phase. Because the acute events leading to diagnosis of CHD or MI had occurred at least 3 weeks before inclusion in this study, selection of patients with a better prognosis compared with a patient population within the early phase of newly diagnosed CHD must be assumed. Furthermore, not all patients were willing or able to participate in the rehabilitation program, and thus the more severely ill patients particularly may be under-represented in this study. The cystatin C level was determined at the end of the rehabilitation program, which was on average 45 days after the acute event or coronary artery revascularization. No association was found between cystatin C level and the time since the acute event. Three-year follow-up information was complete for 85.7% of study participants. However, we have no indication that selective response may have influenced the results, because there was no difference between responders and non-responders of the follow-up with respect to age, gender, history of MI, and diabetes.

**TABLE 2. Polymorphisms in the Cystatin C Gene and Plasma Levels of Cystatin C**

<table>
<thead>
<tr>
<th>Polymorphisms (single and in combination)</th>
<th>Genotype</th>
<th>N</th>
<th>Mean Cystatin-C Plasma Level (SD), mg/L</th>
<th>P (crude)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>82GC Wild-type G/G</td>
<td></td>
<td>659</td>
<td>1.12 (0.33)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/C</td>
<td></td>
<td>313</td>
<td>1.09 (0.36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td></td>
<td>41</td>
<td>0.98 (0.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5GA Wild-type G/G</td>
<td></td>
<td>936</td>
<td>1.12 (0.34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/A</td>
<td></td>
<td>72</td>
<td>0.99 (0.22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td></td>
<td>5</td>
<td>0.92 (0.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4AC Wild-type A/A</td>
<td></td>
<td>660</td>
<td>1.12 (0.33)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/C</td>
<td></td>
<td>314</td>
<td>1.08 (0.36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td></td>
<td>39</td>
<td>0.97 (0.21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>148AG Wild-type G/G</td>
<td></td>
<td>653</td>
<td>1.13 (033)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/A</td>
<td></td>
<td>307</td>
<td>1.06 (0.25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td></td>
<td>49</td>
<td>1.13 (0.70)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Adjusted for age and sex.

**TABLE 3. Association Between Major Haplotype of −82GC, −5GA, and +4GA Polymorphism in the Cystatin C Gene and Plasma Levels of Cystatin C (Multivariate Analysis)**

<table>
<thead>
<tr>
<th>Major Haplotype</th>
<th>β-Coefficient (P)</th>
<th>Hazard Ratio (95 CI) Adjusted for Age and Sex</th>
<th>Hazard Ratio (95 CI) Adjusted for Multiple Covariates*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>−0.042 (P=0.07)</td>
<td>0.81 (0.48–1.38)</td>
<td>0.83 (0.48–1.45)</td>
</tr>
<tr>
<td>None</td>
<td>−0.140 (P=0.01)</td>
<td>0.58 (0.14–2.38)</td>
<td>0.89 (0.21–3.75)</td>
</tr>
</tbody>
</table>

*Adjusted for age, sex, creatinine clearance, body mass index, HDL cholesterol, smoking status, school education, family status, history of diabetes, history of myocardial infarction, clinical score, and hospital site, and NT-proBNP.
Conclusion
The major haplotype −82G/−5G/+4A of the −82GC, −5GA, and +4AC polymorphisms in the cystatin C gene determine plasma levels of cystatin C with persons homozygous for the major haplotype having the highest plasma levels. However, in patients with CHD, the haplotype alone was not associated with prognosis. For the very high plasma levels of cystatin C, possibly associated with poorer prognosis of CHD patients, other, unknown environmental or genetic factors may be of relevance.

Acknowledgments
Dade Behring provided the reagents to measure cystatin C. Otherwise, it had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. We thank Sabine von Karger and Gerlinde Trischler for technical assistance.

References
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