Heme oxygenase-1 gene promoter polymorphism and restenosis following coronary stenting†

Klaus Tiroch*, Werner Koch, Nikolas von Beckerath, Adnan Kastrati, and Albert Schömig

Deutsches Herzzentrum München, 1. Medizinische Klinik Rechts der Isar, Technische Universität München, Lazarettstrasse 36, München D-80636, Germany

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Introduction

Percutaneous coronary intervention has become the mainstay of therapy for coronary artery disease (CAD), and stent implantation efficiently reduces complications and restenosis compared with balloon angioplasty alone. As most technical problems, like vessel recoil or intimal dissection, have been solved during the last years, the major drawback of this technique is restenosis secondary to neointimal hyperplasia. Angiographic restenosis is present in around 30% of cases using bare metal stents and 10–15% for drug eluting stents, with higher rates for complex lesions and for diabetic patients. Previous studies have shown an association of genetic factors with the development of stent restenosis.

A previous study from our institution, comparing the expression level of 2345 genes in restenotic material derived from atherectomy with control specimens, showed a significantly increased expression level of the heme oxygenase-1 (HO-1) gene, suggesting an important role in the process leading to the formation of restenosis. Various oxidative agents induce HO-1 protein production. The HO-1 protein is synthesized by vascular smooth muscle cells and is a rate-limiting enzyme in heme degradation, leading to the generation of free iron and anti-oxidative products like biliverdin and carbon monoxide. Experiments with cell culture and animal models showed that the HO-1 protein exerts potent anti-proliferative and anti-inflammatory effects, and inhibits vascular smooth muscle cell activation. The HO-1 protein attenuates vascular remodelling, mediates endothelial cell apoptosis, and inhibits monocyte transmigration and the development of injury-induced vascular neointima formation.

The human HO-1 gene was mapped to chromosome 22q12. The length of a highly polymorphic (GT)n dinucleotid repeats sequence in the HO-1 gene promoter influences the transcriptional activity. Short (GT)n repeats (n < 25) facilitate upregulation of transcriptional activity of the HO-1 gene. Previous association studies of the HO-1 GT(n) polymorphism divided therefore the alleles into subclasses with shorter and longer alleles. The most frequently used classification was class S including short (n < 25) repeats sequences, and class L including long (n > 25) repeats sequences, leading to SS, SL, and LL genotypes. Angiographic restenosis rate showed no significant difference for the studied genotypes—SS 29.2%, SL 29.5%, and LL genotype 29.6% (P = 0.99). There was no significant difference regarding clinical restenosis (P = 0.28) and combined incidence of death or MI (P = 0.98).

Conclusion This study does not support a clinically relevant association of the HO-1 promoter polymorphism with restenosis and ischaemic events after coronary stenting.

Aims Gene expression analyses, cell culture experiments, animal models, and association studies suggest a protective role of the heme oxygenase-1 (HO-1) protein against restenosis. The length of a polymorphic (GT)n dinucleotid repeats sequence in the HO-1 gene promoter influences the transcriptional activity. We evaluated, whether an association existed between this polymorphism and the incidence of restenosis after coronary stenting.

Methods and results Of the 1807 consecutive patients included in this study, 1357 (75%) patients had 6 months follow-up angiography. Restenosis, the primary endpoint, was defined as angiographic restenosis, diameter stenosis of ≥50%, and clinical restenosis, target vessel revascularization during the first year. The combined 1 year incidence of death and myocardial infarction (MI) was evaluated as secondary endpoint. We divided the alleles similar to previous studies: class S less repeats (<25), and class L more repeats (≥25), leading to SS, SL, and LL genotypes. Angiographic restenosis rate showed no significant difference for the studied genotypes—SS 29.2%, SL 29.5%, and LL genotype 29.6% (P = 0.99). There was no significant difference regarding clinical restenosis (P = 0.28) and combined incidence of death or MI (P = 0.98).

Changes in the manuscript:

1. Corrections in spelling and grammar have been made throughout the text.
2. The introduction and methods sections have been expanded for clarity.
3. The conclusions have been reformulated to reflect the findings more accurately.

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We have also assessed the influence of the HO-1 length polymorphism on the C-reactive protein response to coronary stenting. Previous studies have shown an association of the inflammatory marker C-reactive protein with different cardiovascular events, and studies from our institution have revealed an association of the post-interventional C-reactive protein increase with restenosis following coronary intervention with bare metal stents.27–32

Our study is based on functional data from gene expression analyses from our institute, data from cell culture experiments and animal models, and on data from previous clinical association studies.5–25,33 We evaluated culture experiments and animal models, and on data from expression analyses from our institute, data from cell

**Methods**

**Patients**

The significance of the studied HO-1 (GT)n promoter polymorphism was evaluated in a cohort study comprising 1807 consecutive Caucasian patients with symptomatic CAD, who underwent coronary bare-metal stent implantation at Deutsches Herzzentrum München and 1. Medizinische Klinik Rechts der Isar der Technischen Universität München with pre- and post-interventional measurements of C-reactive protein by a high sensitivity assay as described below. Between January 2000 and February 2002, 2400 patients were screened for inclusion in this study. Of these, 190 patients did not consent for DNA collection and 403 patients lacked either baseline or post-procedural C-reactive protein measurements. Thus, 1807 patients constituted the study cohort. Stent placement and post-stenting therapy were described already in detail.34 Briefly, post-procedural therapy consisted of aspirin 100 mg twice daily, indefinitely, and clopidogrel 75 mg once daily for 4 weeks. Follow-up angiography was routinely scheduled at 6 months post-stenting or whenever the patient complained of anginal symptoms. Creatine kinase levels and ECG changes were assessed systematically over 48 h after the procedure. Clinical events were monitored throughout a 1-year period following the intervention. The data regarding cardiovascular risk factors were obtained during the actual hospitalization or from the patient’s chart. Our study complies with the Declaration of Helsinki, the locally appointed Ethics Committee approved the study, and all patients gave written informed consent for the intervention, follow-up angiography, and genotype determination.

**Measurement of C-reactive protein**

Venous blood samples were collected from each patient at admission and 24 h after coronary stenting and were immediately analysed. C-reactive protein was measured by a high sensitivity assay (Tina-quant®). Its analytic sensitivity (lowest measurable C-reactive protein concentration that can be distinguished from zero) was 0.03 mg/L. The functional sensitivity, i.e. the lowest C-reactive protein concentration that can be reproducibly measured with an interassay coefficient of variation < 10%, is 0.11 mg/L.35–32

**Angiographic assessment**

Computer-based quantitative angiographic analysis was performed using the automated edge-detection system CMS assessing matched views of the target lesions (Medis Medical Imaging Systems, Nuenen, The Netherlands). Angiographic parameters were recorded before and immediately following the intervention, and during the follow-up angiography. Lesion morphology was assessed according to the modified American Heart Association/American College of Cardiology and classified as type A, B1, B2, or C.3 Lesions of types B2 and C were considered complex lesions. Lesion length, reference diameter, minimal lumen diameter, diameter stenosis, and diameter of the maximally inflated balloon during the intervention were measured and assessed for each patient. Late lumen loss was calculated as the difference between the final post-stenting MLD and the MLD measured at follow-up angiography.

**Genetic analysis of the HO-1 gene (GT)n repeat polymorphism**

Genomic DNA was extracted from 200 μL of peripheral blood using commercially available kits (Qiagen, Hilden, Germany, and Roche Molecular Biochemicals, Mannheim, Germany). The GT(n) repeat frequency was assessed using the GeneScan method as previously described.24 We used a 5′ FAM-labelled sense primer (FAM-GCTCTGGAAAGGACAAACTAC) and a 3′ unlabelled antisense primer (GGTGGAGGGAGCAGTATGAC). DNA amplifications were performed by 29 cycles of denaturation at 95°C for 15 s and primer annealing and extension at 68°C for 1 min. We then analysed the PCR products using a capillary sequencer (ABI Prism™ 3100 Genetic Analyser, Applied Biosystems, Foster City, CA). The labelled PCR products with different sizes, depending on the number of GT dinucleotid repeats, were compared with a standard size marker GenoType™ TMA DNA ladder (size range 50–500 bp) (GibcoBRL). The number of (GT)n repeats was estimated using the GeneScan Analysis software (Applied Biosystems).

To control for correct genotype assignment with the GeneScan method, PCR products from 923 samples were further analysed by gel electrophoresis, and their size estimated from comparisons with standard size markers (Roche). The two different methods revealed consistent results.

In addition, representative PCR products, including the samples from patients with the shortest (13 repeats) and the longest repeats (44), and representative samples spanning the entire range of repeats in steps of five (GT)n repeats, were sequenced (Big Dye® Terminator v1.1 Cycle Sequencing Kit, Pat. No. 4336776) and analysed to control for the accuracy of the GeneScan results. Sequencing results were in accordance with the representative GeneScan results.

To control for correct sample handling, genotyping was repeated with the DNA from 20% of the patients. All control experiments revealed identical results when compared with the first genotyping. Two independent operators assessed all GeneScan results as well as all sequencing data. Sequencing data corresponded to the HO-1 sequence from the Pubmed GenBank X14782.

**Study endpoints and definitions**

The primary endpoint of the study was restenosis that was evaluated angiographically and clinically. Angiographic restenosis was defined as a diameter stenosis of ≥ 50% at 6 months follow-up angiography. Clinical restenosis was defined as the necessity for target vessel revascularization (TVR) by percutaneous transluminal coronary angioplasty or coronary arterial bypass grafting due to symptoms or signs of ischaemia in the presence of angiographic restenosis at the site of the intervention during the first year following stenting. Secondary endpoint was the combined 1 year incidence of death from any cause and non-fatal myocardial infarction (MI) after stenting. The diagnosis of MI was based on the presence of new pathologi- cal Q waves or a value of creatine kinase or its MB isoenzyme at least three times the upper limit. Patients with acute MI were those who were admitted for the treatment of acute MI (time period from onset of pain to treatment < 72 h) with percutaneous coronary intervention. Systemic arterial hypertension was defined as systolic blood pressure of 140 mmHg or greater and/or a diastolic blood
pressure of 90 mmHg or greater at least on two separate occasions or anti-hypertensive treatment. Diabetes mellitus was defined in the presence of an active treatment with insulin or an oral antidiabetic agent; for patients on dietary treatment, documentation of an abnormal fasting blood glucose or glucose tolerance test based on the World Health Organization criteria was required for establishing this diagnosis. Persons reporting regular smoking in the prior 6 months were considered as current smokers. Hypercholesterolemia was defined as documented total cholesterol ≥ 240 mg/dL or respective treatment.

**Statistical analysis**

Continuous variables were expressed as mean ± SD and compared by means of the unpaired, two-sided t-test or analysis of variance for more than two groups. The sample size included had an 80% power to detect a significant 25% increase in TVR among LL patients. Discrete variables were expressed as counts or percentages and compared with the $\chi^2$ or Fisher's exact test, as appropriate. Statistical analyses were performed using S-Plus software (Mathsoft Inc., Seattle, Washington). A two-sided $P$-value <0.05 was considered statistically significant.

**Results**

Our population included 1807 consecutive patients after coronary stenting, and 1357 patients (75.1%) had a follow-up angiography 6 months following stent implantation.

The number of repeats ranged from 13 to 44, with the highest prevalence for the GT(23) repeat ($n = 713$) and the GT(30) repeat ($n = 1590$). The allelic distributions showed no significant differences for patients without restenosis and patients with restenosis, as displayed in Figure 1.

Baseline characteristics of our study population are presented in Table 1. No significant differences were observed between the genotype groups with the exception of a significant higher prevalence of hypercholesterolemia and previous coronary artery bypass grafting in the group of patients with the SS genotype compared to the SL genotype. Lesion and procedural characteristics were not significantly different between the SS, SL, and LL genotype groups (Table 2). The C-reactive protein values at baseline and the maximum C-reactive protein level showed no significant difference, and the increase from baseline measured 24 h after the intervention—SS genotype 37.7 mg/L, SL genotype 36.6 mg/L, and LL genotype 31.8 mg/L ($P = 0.09$)—was not significantly different between the studied genotypes.

Major adverse clinical events including death, non-fatal MI, and urgent TVR during the early 30-day period after coronary artery stenting, showed no statistical significant difference for the studied genotypes (Table 3). The combined endpoint of death or MI during the first 30 days was 4.1% for the SS, 3.2% for the SL, and 3.2% for the LL genotype ($P = 0.80$).

The findings of the follow-up angiography are shown in Table 4. Angiographic restenosis was observed in 401 patients (29.6%). The angiographic restenosis rate, our primary endpoint, showed no significant difference between the studied groups—SS genotype 29.2%, SL genotype 29.5%, and LL genotype 29.6% ($P = 0.99$). We also analysed whether the definition of class S (<25 repeats) and class L (≥25 repeats) has an impact on the result. We excluded the GT(23) repeat or included the GT(24) repeat

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**Table 1** Baseline characteristics of patients in the SS (both alleles <25 repeats), SL, and LL (both alleles ≥25 repeats) groups ($n = 1807$)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SS ($N = 197$)</th>
<th>SL ($N = 697$)</th>
<th>LL ($N = 913$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>65.5 ± 10.8</td>
<td>66.2 ± 10.7</td>
<td>65.7 ± 10.2</td>
<td>0.51</td>
</tr>
<tr>
<td>Women</td>
<td>19.8</td>
<td>25.4</td>
<td>24.5</td>
<td>0.27</td>
</tr>
<tr>
<td>Arterial hypertension</td>
<td>52.3</td>
<td>49.5</td>
<td>49.4</td>
<td>0.75</td>
</tr>
<tr>
<td>Diabetes</td>
<td>22.8</td>
<td>28.7</td>
<td>24.8</td>
<td>0.11</td>
</tr>
<tr>
<td>Current smoker</td>
<td>17.3</td>
<td>16.5</td>
<td>19.6</td>
<td>0.26</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>55.8</td>
<td>43.3</td>
<td>46.1</td>
<td>0.008</td>
</tr>
<tr>
<td>Stable angina</td>
<td>59.4</td>
<td>57.0</td>
<td>59.8</td>
<td>0.50</td>
</tr>
<tr>
<td>Unstable angina</td>
<td>23.9</td>
<td>27.8</td>
<td>25.1</td>
<td>0.35</td>
</tr>
<tr>
<td>Acute MI</td>
<td>16.8</td>
<td>15.2</td>
<td>15.1</td>
<td>0.84</td>
</tr>
<tr>
<td>Previous MI</td>
<td>38.1</td>
<td>34.3</td>
<td>36.8</td>
<td>0.47</td>
</tr>
<tr>
<td>Previous CABG</td>
<td>20.3</td>
<td>12.1</td>
<td>14.8</td>
<td>0.01</td>
</tr>
<tr>
<td>C-reactive protein before intervention</td>
<td>3.9</td>
<td>5.1</td>
<td>3.8</td>
<td>0.09</td>
</tr>
<tr>
<td>Number of diseased vessels</td>
<td></td>
<td></td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td>One vessel</td>
<td>23.9</td>
<td>23.0</td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td>Two vessels</td>
<td>34.5</td>
<td>30.8</td>
<td>31.6</td>
<td></td>
</tr>
<tr>
<td>Three vessels</td>
<td>41.6</td>
<td>46.2</td>
<td>45.0</td>
<td></td>
</tr>
</tbody>
</table>

$P < 0.05$ for hypercholesterolemia and previous CABG; $P = $ not significant for all other comparisons. Age is mean ± SD; C-reactive protein in mg/L; other variables are % of patients. CABG, coronary artery bypass grafting.
to the group with shorter alleles, respectively, and evaluated the association with angiographic restenosis. There was no significant association with angiographic restenosis. We therefore concluded that the definitions of class S alleles and class L alleles, and of the SS, SL, and LL genotypes did not have a relevant impact on the association with angiographic restenosis. The clinical restenosis rate was 16.2% for the SS genotype, 19.7% for the SL genotype, and 20.9% for the LL genotype, showing no statistical significant difference (P = 0.32) (Table 5).

Table 5 shows other adverse events occurring within 1 year after the procedure without significant difference of their distributions among the genotypes. Notably, the combined 1 year incidence of death or MI, our secondary endpoint, was similar regarding the studied genotypes (P = 0.98).

**Discussion**

This study was based on previous data revealing an increased expression of the HO-1 gene in restenotic material compared to control specimen and on functional data derived from cell culture experiments and animal models. These data have shown an important role of the HO-1 gene and its protein product for the inhibition of inflammation induced proliferation and neointimal growth. Further, the length of the highly polymorphic (GT)n
The main result of our study, based on a large number of 1807 consecutive patients, is that the (GT)n dinucleotide repeats length polymorphism located in the promoter region of the HO-1 gene is not associated with the development of restenosis and major adverse clinical events following coronary stenting.

We have reported that no correlation existed between basal C-reactive protein levels and restenosis, but found a correlation between increase in C-reactive protein after the procedure and restenosis. The present study includes all patients analysed previously. Consistent with this association of restenosis and C-reactive protein, our study found negative associations for the HO-1 polymorphism with the risk of restenosis as well as with the post-interventional C-reactive protein levels. The fact that the HO-1 promoter polymorphism was associated neither with the incidence of restenosis nor with post-interventional C-reactive protein levels further supports our conclusion.

Strengths of our study were the large series of consecutive patients, a follow-up angiography rate performed in more than 75% of the included patients, the computer-based angiographic assessment, and the use of different measures to assure correct genotyping. The genotype distribution is similar to the genotype distributions described previously in Caucasian or Chinese populations, and the angiographic and clinical restenosis rate in this study is comparable to recently reported studies. The negative result was independent of the definition of class S or class L alleles and the respective SS, SL, and LL genotypes, excluding a bias created by the definition of the genotype-based study groups.

Given the controversial data of previous studies, our results are important for defining the role of the HO-1 polymorphism for the development of restenosis and major adverse clinical events. This study does not support a clinically relevant association of the (GT)n length polymorphism in the HO-1 gene promoter with restenosis and ischaemic events after coronary stenting.

**Limitations**

One limitation of this study might be related to the incomplete angiographic follow-up. About 25% of the patients did not have coronary angiography at 6 months mostly due to their refusal. However, the main conclusions of this study are probably not influenced by this limitation, because the information on clinical restenosis, the co-primary endpoint, was available in all patients and showed results concordant with those of follow-up angiography.

Another limitation is related to the power of the study. The sample size included had an 80% power to detect a significant 25% increase in TVR among LL patients, as described in previous studies. It is obvious that this study has less power to detect differences smaller than the above assumption.

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**Conflict of interest**: none declared.

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