Genetic Analyses of Heme Oxygenase 1 (HMOX1) in Different Forms of Pancreatitis

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Abstract

Background: Heme oxygenase 1 (HMOX1) is the rate limiting enzyme in heme degradation and a key regulator of inflammatory processes. In animal models the course of pancreatitis was ameliorated by up-regulation of HMOX1 expression. Additionally, carbon monoxide released during heme breakdown inhibited proliferation of pancreatic stellate cells and might thereby prevent the development of chronic pancreatitis (CP). Transcription of HMOX1 in humans is influenced by a GT-repeat located in the promoter. As such, HMOX1 variants might be of importance in the pathogenesis of pancreatitis.

Methods: The GT-repeat and SNP rs2071746 were investigated with fluorescence labelled primers and by melting curve analysis in 285 patients with acute pancreatitis, 208 patients with alcoholic CP, 207 patients with idiopathic/hereditary CP, 147 patients with alcoholic liver cirrhosis, and in 289 controls, respectively. GT-repeat analysis was extended to a total of 446 alcoholic CP patients. In addition, we performed DNA sequencing in 145 patients with alcoholic CP, 138 patients with idiopathic/hereditary CP, 147 patients with alcoholic liver cirrhosis, and 151 controls. Exon 3 screening was extended to additional patients and controls.

Results: S- and L-alleles of the GT-repeat, genotypes and alleles of SNP rs2071746 and non-synonymous variants detected by sequencing were found with similar frequencies in all groups.

Conclusions: Although functional data implicate a potential influence of HMOX1 variants on the pathogenesis of pancreatitis, we did not find any association. As rare non-synonymous HMOX1 variants were found in patients and controls, it is rather unlikely that they will have functional consequences essential for pancreatitis development.

Introduction

Acute pancreatitis (AP) is a potentially life threatening disease presenting with a wide clinical spectrum that ranges from mild discomfort to multi organ failure [1]. In the Western world, the leading causes of AP are chole(doch)olithiasis and alcohol abuse. Apart from the p.N34S SPINK1 variant, no other genetic association with AP has been confirmed so far [2]. Chronic pancreatitis (CP) is a relapsing inflammatory disease resulting in a permanent impairment of exocrine and endocrine organ function in many cases. In industrialised countries, chronic alcohol abuse is the major underlying cause while nicotine abuse is an important contributing factor [3]. Although several genetic associations have been described for idiopathic and hereditary CP (ICP, HP) little is known about genetic alterations that

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contribute to the pathogenesis of AP and alcoholic CP (ACP) [4–10].

There are 2 functional heme oxygenase isoforms, the inducible HMOX1 (also designated as HO-1, OMIM*141250) and the constitutively expressed HMOX2 [11]. HMOX1 is a key regulator of inflammatory processes as moderate over-expression of HMOX1 protects cells, whereas excessive HMOX1 expression is harmful [12]. Cellular HMOX1 content is transcriptionally regulated and gene expression is induced by many different stimuli such as heavy metals, inflammation, UV radiation, oxidative stress and even by HMOX1 itself after translocalisation to the nucleus [13]. Gene transcription in humans is modulated by the length of a dinucleotide GT-repeat in the promoter classified in short alleles and long alleles [14]. Previous studies demonstrated an inducible elevation of HMOX1 activity in presence of S-alleles. For example HMOX1 mRNA content and oxidative stress induced HMOX1 enzymatic activity are significantly higher in S-allele carriers [15].

In animal models of experimental pancreatitis, expression of HMOX1 is up-regulated in pancreatic islet and acinar cells and HMOX1 as well as carbon monoxide (CO) act protective [16–18]. Moreover, HMOX1 induction improves outcome after pancreas transplantation and ameliorates microcirculatory derangements after ischemia and reperfusion [19,20]. For the development of CP, fibrotic remodelling of the pancreatic parenchyma is an essential step, with a crucial role for pancreatic stellate cells (PSC) [21]. Of note, PSC proliferation is inhibited by CO and endogenous CO is mainly produced during the breakdown of heme by microsomal HMOXs [22].

As such, it is reasonable to portend that different GT-repeat alleles or other HMOX1 genetic alterations contribute to the pathogenesis of different pancreatitis phenotypes. To investigate the role of HMOX1 alterations in pancreatitis, we extensively screened the GT-repeat, SNP rs2071746, and the coding sequence in up to 446 patients with different forms of pancreatitis, 147 patients with alcoholic liver cirrhosis (ALC) and up to 413 healthy controls.

Materials and Methods

Patients and Controls

The study was approved by the medical ethical review committee of the University of Leipzig, Germany (Approval: 376-11-12122011). All patients gave written informed consent. AP was diagnosed and categorised according to the Atlanta classification [1]. We categorised patients into a group with a mild disease course (only local complications) and a group with severe disease course (additionally systemic complications). Diagnosis of CP was based on two or more of the following findings: Presence of a history of recurrent pancreatitis or recurrent abdominal pain typical for CP, pancreatic calcifications and/or pancreatic ductal irregularities revealed by endoscopic retrograde pancreatography or by magnetic resonance imaging of the pancreas and/or pathological sonographic findings. ACP was defined and categorised according to the literature in short repeats (S<25) and long repeats (L≥25) [23].

Melting curve analysis of rs2071746

We performed PCR in the LightCycler 480 instrument (Roche Diagnostics) under the following conditions (volumes see below): initial denaturation at 95°C for 3 minutes followed by 45 cycles with denaturation at 95°C for 5 seconds, annealing at 55°C for 20 seconds, primer extension at 72°C for 20 seconds. Primers had the following oligonucleotide sequences: Forward primer 5'-GAGGGTCTGCAGGTTTCTCAGA-3', reverse Primer 5'-TGGAGAGGACACGTCA-TATG-3'. We loaded PCR products together with a size standard onto an ABI 3100 fluorescence sequencer (Applied Biosystems) for fragment analysis and determined the length of the amplified PCR product as the number of GT-repeats. We classified the products containing GT-repeats according to the literature in short repeats (S<25) and long repeats (L≥25) [23].
included the following steps: 95°C for 5 seconds, 40°C for 20 seconds and an increase to 80°C at a 0.29°C/s ramp rate.

**Polymerase Chain Reaction and DNA sequencing**

We extracted genomic DNA from peripheral blood leukocytes and performed PCR using 0.75 U AmpliTaq Gold polymerase (Applied Biosystems), 400 μM dNTPs, 1.5 mM MgCl₂ and 0.1 μM of each primer in a total volume of 25 μl. Cycle conditions were as follows: an initial denaturation for 12 minutes at 95°C followed by 40 cycles of 20 seconds denaturation at 95°C, 40 seconds annealing at specific temperatures, 90 seconds primer extension at 72°C and a final extension for 2 minutes at 72°C in an automated thermal cycler. Oligonucleotide sequences and annealing temperatures of the primers are listed in Table S1.

We digested PCR products with shrimp alkaline phosphatase (USB) and exonuclease I (GE Healthcare) and performed cycle sequencing using BigDye terminator mix (Applied Biosystems). We purified reaction products with ethanol precipitation and loaded them onto an ABI 3100 fluorescence sequencer (Applied Biosystems). Mutations are described according to the nomenclature recommended by the Human Genome Variation Society (http://www.hgvs.org/mutnomen) following a common consensus with the mutation numbering which defines the A of the ATG start codon as nucleotide +1.

**Statistics**

We tested the significance of the differences between variant frequencies in affected individuals and controls by two-tailed Fisher’s Exact test. P-values were calculated using GraphPad Prism (v 4.03). For SNPs we utilised a dominant model, defined as AA vs. AG+GG (e.g. for c.736+226A>G, rs2269533), for calculations and considered p-values <0.05 to be of statistical significance. In addition, calculations were performed following a recessive model (AA+AG vs. GG) and for allele frequencies. We used the first allele in the variant description as the major allele (example above for c.736+226A>G, rs2269533). The p-values are shown without correcting for multiple testing.

**Results**

**Fragment length analyses of the promoter GT-repeat**

Our analysis of the GT-repeat revealed repeats ranging from 12 to 40 G Ts. In all groups we detected alleles with 23 (AP 19.7%, ACP 24.2%, ICP/HP 21.1%, ALC 22.8%, controls 19.7%) and 30 repeats (AP 42.5%, ACP 45.6%, ICP/HP 48.1%, ALC 41.5%, controls 43.3%) with highest frequencies in accordance with previous reports [23]. In AP patients, frequencies of S- and L-alleles were similar to frequencies obtained in controls (S-allele: 173/578, 29.9%; Genotype: AA 25/80, 31.3%; AT 34/80, 42.5%) disease course no difference was obtained in the AP group compared to controls (all p-values not significant).

| Alleles | Patients | Controls | p-Value |
|---------|----------|----------|---------|
| AP      |          |          |         |
| S       | 189/570 (33.2%) | 173/578 (29.9%) | n.s     |
| L       | 381/570 (66.8%) | 405/578 (70.1%) |         |
| ACP     |          |          |         |
| S       | 286/892 (32.1%) | 173/578 (29.9%) | n.s     |
| L       | 606/892 (67.9%) | 405/578 (70.1%) |         |
| ICP/HP  |          |          |         |
| S       | 125/412 (30.3%) | 173/578 (29.9%) | n.s     |
| L       | 287/412 (69.7%) | 405/578 (70.1%) |         |
| ALC     |          |          |         |
| S       | 91/294 (31%) | 173/578 (29.9%) | n.s     |
| L       | 203/294 (69%) | 405/578 (70.1%) |         |

S-alleles were defined as <25 GT-repeats, whereas L-alleles represented ≥25 GT-repeats. Abbreviations: S = S-allele, L = L-alleles, n.s. = not significant. doi:10.1371/journal.pone.0037981.t001

| Variant | Genotype | Patients | Controls | p-Value |
|---------|----------|----------|----------|---------|
| g.4613A>T rs2071746 | AP | | |
| AA      | 85/285 (29.8%) | 96/289 (33.2%) | n.s     |
| AT      | 143/285 (50.2%) | 142/289 (49.1%) |       |
| TT      | 57/285 (20%) | 51/289 (17.6%) |         |
| ACP     | | | |
| AA      | 62/208 (29.8%) | 96/289 (33.2%) | n.s     |
| AT      | 107/208 (51.4%) | 142/289 (49.1%) |       |
| TT      | 39/208 (18.8%) | 51/289 (17.6%) |         |
| ICP/HP  | | | |
| AA      | 73/207 (35.3%) | 96/289 (33.2%) | n.s     |
| AT      | 104/207 (50.2%) | 142/289 (49.1%) |       |
| TT      | 30/207 (14.5%) | 51/289 (17.6%) |         |
| ALC     | | | |
| AA      | 51/147 (34.7%) | 96/289 (33.2%) | n.s     |
| AT      | 68/147 (46.3%) | 142/289 (49.1%) |       |
| TT      | 28/147 (19%) | 51/289 (17.6%) |         |

Abbreviations: n.s. = not significant. doi:10.1371/journal.pone.0037981.t002
DNA sequencing

By DNA sequencing of the complete coding region, we found five common HMOX1 variants (c.144+4T>G, rs17855925; c.143–19C>T, rs17879606; c.376+52delTinsTGCTGTCTGACT, rs17882597; c.376+226A>G, rs2269533; c.736+270T>C, rs2269534) in patients and controls (Table S2). Apart from SNP rs2269534 in ACP patients, frequencies of all SNPs were similar in patients and controls and the distribution showed no statistical significant difference when allele frequencies and genotypes were compared. In ACP patients allele frequencies of SNP rs2269534 differed significantly between patients (7.5%) and controls (11.7%) (p-value 0.04, OR 1.5, 95% CI 1.02–2.13), but the association did not withstand Bonferroni correction (p-value 0.2).

We identified non-synonymous variants and variants with a minor allele frequency <5% in 25/145 (17.2%) ACP patients and in 27/151 (17.9%) controls (Table 3). Non-synonymous variant c.19G>C (p.D7H, rs2071747) was detected in similar frequencies in ACP patients (14/145, 9.7%) and controls (15/151, 9.9%). We found variants c.379G>T (p.E127X) and c.577C>T (p.P193S) in patients only (1/145, 0.7%; 2/145, 1.4%), whereas we detected variant c.101T>G (p.M34T) in one control only (1/151, 0.7%). None of the variants showed a statistical significant difference of their frequencies in patients and controls.

In the cohort of ICP/HP patients, only one patient (1/138, 0.7%) carried a non-synonymous variant (c.407G>A, p.R136H) apart from c.19G>C (11/138, 7.2%). In patients with ALC, no variant c.19G>C (11/138, 7.2%). In patients with ALC, no variant carried a non-synonymous variant other than c.19G>C (11/147, 7.5%). Overall, we observed no significant difference in the distribution of variants in both patient groups compared to controls.

To elucidate the role of non-synonymous exon 3 variants (c.379G>T, c.407G>A, and c.577C>T), we extended our analyses (Table 4). Subsequently, we found variant c.379G>T in 1/446 (0.2%) ACP patients, but not in ICP/HP patients or controls. Variant c.577C>T in 2/446 (0.4%) ACP patients and in 2/248 (0.8%) ICP/HP patients compared to 2/413 (0.5%) controls, and variant c.407G>A in 1/248 (0.4%) ICP/HP patients, but not in 413 controls. In addition, we identified a hitherto undescribed variant c.473C>T (p.P158L) in one control subject. In summary, none of the variants alone or combined showed a significant difference in their distribution between patients and controls (Table 4).

Discussion

The current study investigates the influence of genetic HMOX1 alterations in different types of pancreatitis since there is evidence that HMOX1 induction might influence the development and the course of AP and the pathogenesis of CP by inhibition of PSC proliferation. The finding of a genetic association with different pancreatitis forms would efforts study to efforts efforts HMOX1 induction by drugs or by application of hemin in a clinical setting. However, we did not find any association between the HMOX1 GT-repeat and any of the investigated pancreatitis phenotypes. We observed a trend towards an enrichment of S-alleles in AP and ACP, but this finding is counterintuitive as the S-allele has been associated with more HMOX1 activity and thereby probably protects against the development of pancreatitis [15].

| Table 3. Rare variants identified by sequencing of HMOX1 in alcoholic chronic pancreatitis (ACP), idiopathic/hereditary chronic pancreatitis (ICP/HP), and alcoholic liver cirrhosis (ALC) patients and controls and their location within HMOX1. |
| --- |
| **Variant** | **Location** | **ACP** | **ICP/HP** | **ALC** | **Controls** | **P-Value** |
| c.−120A>T | 5’-UTR | 0/145 | 0/138 | 0/147 | 1/151 (0.7%) | n.s. |
| c.−88T>C | 5’-UTR | 0/145 | 0/138 | 0/147 | 1/151 (0.7%) | n.s. |
| c.−13C>T (rs9282701) | 5’-UTR | 1/145 (0.7%) | 0/138 | 1/147 (0.7%) | 1/151 (0.7%) | n.s. |
| c.19G>C (rs2071747), p.D7H | Exon 1 | 14/145 (9.7%) | 11/138 (7.2%) | 11/147 (7.5%) | 15/151 (9.9%) | n.s. |
| c.101T>G, p.M34T | Exon 1 | 0/145 | 0/138 | 0/147 | 1/151 (0.7%) | n.s. |
| c.23+28, 29delC | Intron 1 | 1/145 (0.7%) | 1/138 (0.7%) | 1/147 (0.7%) | 0/151 | n.s. |
| c.23+91G>A | Intron 1 | 1/145 (0.7%) | 3/138 (2.2%) | 0/147 | 2/151 (1.3%) | n.s. |
| c.23+214C>T | Intron 1 | 0/145 | 2/138 (1.5%) | 2/147 (1.4%) | 1/151 (0.7%) | n.s. |
| c.144+206delTG | Intron 2 | 0/145 | 0/138 | 1/147 (0.7%) | 1/151 (0.7%) | n.s. |
| c.144+246C>T | Intron 2 | 0/145 | 0/138 | 0/147 | 1/151 (0.7%) | n.s. |
| c.144+272C>T | Intron 2 | 0/145 | 0/138 | 1/147 (0.7%) | 0/151 | n.s. |
| c.234C>T, p.(= =) | Exon 3 | 0/145 | 0/138 | 0/147 | 1/151 (0.7%) | n.s. |
| c.330C>T, p.(= =) | Exon 3 | 1/145 (0.7%) | 0/138 | 0/147 | 1/151 (0.7%) | n.s. |
| c.379G>A, p.E127X | Exon 3 | 1/145 (0.7%) | 0/138 | 0/147 | 0/151 | n.s. |
| c.407G>A, p.R136H | Exon 3 | 0/145 | 1/138 (0.7%) | 0/147 | 0/151 | n.s. |
| c.577C>T, p.P193S | Exon 3 | 2/145 (1.4%) | 0/138 | 0/147 | 0/151 | n.s. |
| c.621C>T, p.(= =) | Exon 3 | 1/145 (0.7%) | 0/138 | 0/147 | 0/151 | n.s. |
| c.736+322G>A | Intron 4 | 0/145 | 1/138 (0.7%) | 0/147 | 0/151 | n.s. |
| c.736+331G>A | Intron 4 | 0/145 | 0/138 | 0/147 | 1/151 (0.7%) | n.s. |
| c.*71C>G | 3’-UTR | 0/145 | 2/138 (1.5%) | 0/147 | 0/151 | n.s. |
| c.*149A>G | 3’-UTR | 3/145 (2.1%) | 1/138 (0.7%) | 1/147 (0.7%) | 0/151 | n.s. |

Abbreviations: n.s. = not significant.
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Table 4. Frequency data of exon 3 variants after extension of screening in alcoholic chronic pancreatitis (ACP) and idiopathic/hereditary chronic pancreatitis (ICP/HP) patients and in control subjects.

| Variant Location | Patients | Controls | p-Value |
|------------------|----------|----------|---------|
| c.145→45G>T     | Intron 2 | 1/446 (0.2%) | 0/413 | n.s |
| c.330C>T, (p.(=)) | Exon 3 | 2/446 (0.4%) | 2/413 (0.5%) | n.s |
| c.379G>T, p.E127X | Exon 3 | 1/446 (0.2%) | 0/413 | n.s |
| c.473C>T, p.I158L | Exon 3 | 0/446 | 1/413 (0.2%) | n.s |
| c.577C>T, p.P193S | Exon 3 | 2/446 (0.4%) | 2/413 (0.5%) | n.s |
| c.621C>T, (p.(=)) | Exon 3 | 2/446 (0.4%) | 0/413 | n.s |

ICP/HP

| Variant Location | Patients | Controls | p-Value |
|------------------|----------|----------|---------|
| c.407G>A, p.R136H | Exon 3 | 1/248 (0.4%) | 0/413 | n.s |
| c.577C>T, p.P193S | Exon 3 | 2/248 (0.8%) | 2/413 (0.5%) | n.s |

Abbreviations: n.s. = not significant.

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In earlier studies, the GT-repeat length in the HMOX1 promoter has been associated with several human diseases. To cite coronary artery disease patients as an example, patients with S-alleles had a higher risk at restenosis after coronary stenting [24]. Apart from coronary artery disease patients as an example, patients with S-alleles had a higher risk at restenosis after coronary stenting [24]. Apart from that, previous studies implied a potential influence of the AA-genotype of SNP rs2071746 (g.4613A>G). Apart from that, previous studies implied a potential influence of the AA-genotype of SNP rs2071746 (g.4613A>G) on the risk for restenosis after coronary stenting [24]. Apart from that, previous studies implied a potential influence of the AA-genotype of SNP rs2071746 (g.4613A>G) on the risk for restenosis after coronary stenting [24].

HMOX1 repeat variants the coding region has not been investigated in hypertension and coronary artery disease patients as an example, patients with S-alleles had a higher risk at restenosis after coronary stenting [24]. Apart from that, previous studies implied a potential influence of the AA-genotype of SNP rs2071746 (g.4613A>G) on the risk for restenosis after coronary stenting [24].

In ICP/HP several genetic associations have been described with rare variants over the last years [4–10]. To rule out an association with rare variants over the last years [4–10], we were not able to demonstrate differences of HMOX1 alterations in patients that were smokers compared to non-smokers (data not shown). Noteworthy, the number of individuals in that smoking habits were available was rather small (n = 65) and as such these results have to be interpreted with caution.

In conclusion, our results implicate that HMOX1 variants are unimportant in different forms of pancreatitis and ALC. Notably, HMOX1 is induced by different stimuli and the impact of genetic variants might be minor for a substantial decrease or increase of HMOX1 expression in AP, CP or ALC. Although data obtained in experimentally induced AP picture HMOX1 as a potential target in pancreatitis, we cannot further support this view on the basis of our genetic case control study. Nevertheless, pharmacologic approaches influencing HMOX1 activity might have a beneficial effect even in humans.

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Author Contributions

Conceived and designed the experiments: JR KK SW MJ. Performed the experiments: JR KK SW MJ PK CR. Analyzed the data: JR KK SW MJ PK CR. Contributed reagents/materials/analysis tools: DS RS MH H. Witt JPHD. Wrote the paper: JR SW MJ H. Witt JPHD.

Study limitations

Our study has some limitations. The control group used for comparison are blood donors. As such this introduces potential bias because ALC, ACP or other pancreatitis forms might be present among blood donors. We surmise that the contribution of such a bias is rather small. In Germany all blood donors are routinely screened for elevated liver enzymes and persons with chronic alcohol abuse are not suitable to donate blood. Lastly, the number of ALC patients is relatively small which limits the power of this part of our study.

In conclusion, our results implicate that HMOX1 variants are unimportant in different forms of pancreatitis and ALC. Notably, HMOX1 is induced by different stimuli and the impact of genetic variants might be minor for a substantial decrease or increase of HMOX1 expression in AP, CP or ALC. Although data obtained in experimentally induced AP picture HMOX1 as a potential target in pancreatitis, we cannot further support this view on the basis of our genetic case control study. Nevertheless, pharmacologic approaches influencing HMOX1 activity might have a beneficial effect even in humans.

Supporting Information

Table S1 Oligonucleotide sequences of the primers used for PCR (upper section), DNA-sequencing (lower section), and their annealing temperatures in °C. Abbreviations: PCR = polymerase chain reaction, SEQ = sequencing, F = forward, R = reverse.

(DOCX)

Table S2 Frequencies of common intronic variants in ACP, ICP/HP, and ALC patients. P-values are given for calculations in a dominant model (defined as [e.g. for c.144+4T>C]: TT vs. TC+CC). In addition a recessive model was computed and allele frequencies were compared. Only for variant c.736+270T>C allele frequencies differed significantly between the ACP patients and controls (P-value = 0.041, OR 1.5, 95% CI 1.02–2.13). However, the p-value did not withstand Bonferroni correction (P-value = 0.2). Abbreviations: het. = heterozygous, hom. = homozygous, n.s. = not significant, OR = odds ratio. * p-value given for comparison between ACP patients and controls (before Bonferroni correction).

(DOCX)

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