TLR3 Gene Polymorphisms and Liver Disease Manifestations in Chronic Hepatitis C

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Phenotypes of liver disease due to chronic hepatitis C virus (HCV) infection show a wide range of variations in terms of histological manifestations and the clinical outcome. Sensing of viral double-stranded RNA (dsRNA) by Toll-like receptor 3 (TLR3) is likely involved in early pathogen detection and the host response to viral infection. This study analyzed epidemiological and clinical data from a total of 137 patients with chronic HCV infection with regard to two polymorphic positions within the TLR3 gene: rs5743305 (T/A) is located within the promoter region and might affect transcriptional activity, rs3775291 (C/T) is a non-synonymous single nucleotide polymorphism (SNP) located within exon 4 and the variant receptor has been shown to be functionally impaired. TLR3 promoter and the exon 4 variations were not found to be associated with TLR3 gene expression in peripheral blood mononuclear cells (PBMCs). In the liver, however, a tendency of higher TLR3 gene expression was found for exon 4 TT genotypes. Both variations were not found to be associated with clinical parameters of chronic disease. On the other hand, an analysis of the TLR3 exon 4 genotype distribution with respect to HCV subtype revealed an absence of TT genotype among HCV subtype 1a infected individuals. This study thus failed to reveal any association of the two SNPs under investigation with clinical parameters of chronic hepatitis C. However, data argue for a functional relevance of the exon 4 SNP in terms of conferring a different susceptibility towards HCV subtype infection. J. Med. Virol. 81:1204–1211, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: single nucleotide polymorphism (SNP); hepatitis C virus (HCV); Toll-like receptor (TLR); HCV genotypes; T-976A; L412F

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped single-stranded plus-sense RNA virus that causes acute and often chronic hepatitis [Choo et al., 1989]. Currently, an estimated 3% of the world's population—about 210 million people—is infected with HCV [Shepard et al., 2005]. The natural outcome of infection ranges from silent to self-limited to chronic [Micallef et al., 2006]. The natural course of chronic disease is also highly variable and ranges from asymptomatic to mild disease to cirrhosis and hepatocellular carcinoma (HCC) and HCV-related co-diseases [Scheff, 2002]. The complete pattern of host responses to HCV infection is still unclear. However, the HCV genome encodes regions of extensive secondary double-stranded RNA (dsRNA) structure, and the viral replication cycle includes dsRNA as an intermediate; both entities are assumed to be sensed by pattern recognition receptors (PRRs) during infection [Gale and Foy, 2005].

PRRs comprise membrane Toll-like receptors (TLRs), which are either expressed on the cell surface or on endosomal–lysosomal membranes, and cytosolic receptors such as nucleotide-binding oligomerization domain (NOD)-like receptors and the RNA-helicase family [reviewed in Akira et al., 2006; Takeuchi and Akira, 2007; Seki and Brenner, 2008]. Viral dsRNA is a ligand of TLR3 which is expressed within the endosomal compartment of conventional dendritic cells (cDCs) [Alexopoulos et al., 2001; Matsumoto et al., 2004]. Upon phagocytosis of infectious material and signaling through TLR3, DCs become activated. In the liver,
TLR3 is expressed by Kupffer cells, natural killer (NK) cells, and by hepatocytes [Seki and Brenner, 2008]. TLR3 is also expressed on a variety of epithelial cells, for example, biliary or intestinal epithelium; unlike DCs, epithelial cells appear to express TLR3 on the cell surface [Akira et al., 2006]. More recent studies point, in addition, to a co-stimulatory role of TLR3 on human T lymphocytes [Tabiasco et al., 2006; Wesch et al., 2006].

TLR3 ligand-binding leads to the activation of the transcription factors interferon-regulatory factor-3 (IRF-3) and nuclear factor-xB (NF-xB) and, finally, to the induction of interferon-β (IFN-β) and proinflammatory cytokines, respectively [Santoro et al., 2003; Matsumoto et al., 2004]. In contrast to other members of TLR which associate with a common adapter molecule, myeloid differentiation factor 88 (MyD88), the TLR3 pathway depends on another adaptor protein, the TIR-domain containing adaptor inducing IFN-β (TRIF) [reviewed in Akira et al., 2006; Seki and Brenner, 2008; Vercammen et al., 2008]. IFN-β, in turn, activates several other genes, including 2′-5′-oligoadenylate synthetases, protein kinase R, Mx GTPase, and P56, which contribute to an antiviral effect via the inhibition of protein synthesis or viral replication [Vercammen et al., 2008].

The completion of the human genome project opened up the opportunity to dissect complex human traits and to understand basic pathways of health and disease. Population-based association studies are very useful for examining genes with a role in common multifactorial diseases that have a strong environmental component [Risch, 2000]. These studies often estimate the risk of developing a certain disease in carriers and non-carriers of particular genetic variations especially single nucleotide polymorphisms (SNPs). Genetic variations in the TLRs and their signaling molecules have been correlated with susceptibility to various diseases including sepsis or malignancies [Cook et al., 2004; Akira et al., 2006; El-Omar et al., 2008; Fukata and Abreu, 2008] and to vaccination efficiency [Dhiman et al., 2008].

The human TLR3 gene contains SNPs with relevant minor allele frequencies (MAFs) for the Caucasian population. Whereas SNPs within the promoter region might affect transcriptional activity (e.g., rs5743305 (T-976A)), an amino acid exchange from leucine to phenylalanine at position 412 (rs3775291 (L412F)) has been shown to be associated with a functional impairment of the molecule in terms of mediating signaling [Ranjith-Kumar et al., 2007]. This study aimed at investigating whether genetic variations within the TLR3 promoter and the TLR3 protein are associated with severity of HCV infection.

MATERIALS AND METHODS

Patients

A total of 137 consecutive out-patients, mainly Caucasians, with chronic hepatitis C who consulted the Liver Unit of the Department of Gastroenterology and Endocrinology at the University Medical Center, Göttingen between 1993 and 2006 were enrolled. Chronic HCV infection was diagnosed by the detection of HCV-specific antibodies and by HCV RNA in patients sera using a highly sensitive nested RT-PCR over a period of at least 6 months as described [Mihm et al., 1996a]. Viral genotypes were determined using the Innolipa HCV II line probe assay (Innogenetics, Ghent, Belgium). As part of a routine clinical evaluation, liver biopsy procedures were performed. Liver disease was confirmed histopathologically as described elsewhere [Mihm et al., 1997]. Patients with hepatitis B virus (HBV) or hepatitis A virus (HAV) co-infections and those with continued alcohol or other drug abuse were excluded. The study was approved by the local ethical committee and conformed to the ethical guidelines of the 2000 Declaration of Helsinki. Patients gave their informed consent.

Histopathological Evaluation

Liver biopsies were taken from patients before beginning of therapy for histopathological evaluation. In brief, sections (5–10 μm) from formalin-fixed and paraffin-embedded liver tissue samples were stained with hematoxylin-eosin, trichrome, and Prussian blue reaction. According to Desmet et al. [1994], necro-inflammatory changes (grading) and architectural alterations (staging) were scored separately. Other lesions typical for hepatitis C such as the steatosis degree, portal lymphoid aggregates, or bile duct damage were studied additionally, the definition of each is given elsewhere [Mihm et al., 1997].

Isolation of Genomic DNA and SNP Genotyping

Genomic DNA (gDNA) was purified from peripheral blood mononuclear cell (PBMC) samples by means of QIAamp DNA Mini Kit following the blood and body fluid spin protocol (Qiagen, Hilden, Germany) or, when PBMCs were not available, from 2 ml of serum using QIAamp DNA Blood Midi Kit (Qiagen). The concentration and the purity of gDNA isolated from PBMCs, but not from serum due to the little amount of cells, were determined photometrically by reading the absorbance levels at 260 and 280 nm. An electrophoresis using a 0.6% agarose gel was used to assure the integrity of gDNA.

Genotyping of the variant positions rs5743305 and rs3775291 was performed in the sequence detection system Step One-Plus (Applied Biosystems, Darmstadt, Germany) using the commercially available TaqMan genotyping assays C_393058_10 and C_1731425_10, respectively, with 4 ng PBMCs-derived gDNA or an aliquot corresponding to 6.7 μl serum in 10 μl reactions according to the supplier’s instructions.

Isolation of Total Cellular RNA and Quantitation of Gene Expression

Where available, total cellular RNA was prepared by CsCl density gradient centrifugation from freshly
isolated PBMC and homogenized liver tissue samples from a total of 34 and 48 hepatitis C patients, respectively. PBMCs had been isolated from approximately 30 ml of heparinized peripheral blood samples by Ficoll density centrifugation and from homogenized liver tissue essentially as described [Mihm et al., 1996b]. An amount of 1 mg of total cellular RNA was reverse transcribed by using random hexamers (6 M) for priming. Quantitation of gene expression was performed in a total volume of 10 µl by real-time RT-PCR in a Step One-Plus sequence detection system (Applied Biosystems, Darmstadt, Germany) using commercially available TaqMan gene expression assays for the quantitation of TLR3 (Hs00152933m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905m1) transcripts (Applied Biosystems, Darmstadt, Germany).

**Statistical Analysis**

To avoid bias, data were stratified by age (<40 years, >40 years), sex, and HCV subtypes. To compare individual genotypes, linear, or logistic regression models were applied. To compare individual genotypes between HCV subtypes Cochran–Mantel Haenzel-test was performed after stratification for sex and age. The local level of significance was set to a screening level of \( P = 0.05 \). All calculations were performed with SAS 9.2 or PC STATISTIK software package version 4.0 (Hoffmann-Software, Giessen, Germany).

**RESULTS**

A total of 137 patients with a diagnosis of chronic HCV infection were included. Representative for a European sample, most of the patients were infected with HCV subtype 1b, followed by those who are infected with virus subtypes 1a and 3a. Before the start of a therapy, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and \( \gamma \)-glutamyl transferase (\( \gamma \)-GT) activities were recorded. At the same time, liver biopsy specimens were taken and evaluated histopathologically. Five criteria were examined, including the activity of hepatitis, the degree of fibrosis, the degree of steatosis, the presence of portal lymphoid aggregates, and presence of bile duct damage.

All patients were genotyped for two bi-allelic SNPs within the TLR3 gene: rs5743305 (T/A), which is located at position 976 within the promoter region (relative to the transcriptional start site), and rs3775291 (C/T), which is a non-synonymous SNP within exon 4 and causes an amino acid exchange from leucine to phenylalanine at position 412 of the protein molecule. For both SNPs, genotype distribution and MAF were close to that given for Caucasians in public databases (Tables I and II). For the patients as a whole, no deviation from Hardy–Weinberg equilibrium was found (Tables I and II). Epidemiological analysis revealed no significant relationship between genotypes and both gender and age (Tables I and II).

To investigate whether the TLR3 promoter variants are associated with differences in TLR3 gene expression, the number of TLR3 transcripts was quantified both in PBMC and in liver tissue samples from a total of 34 and 48 hepatitis C patients, respectively (Fig. 1). Data were related to TLR3 promoter genotypes and, as a control, to TLR3 exon 4 genotypes, for which effects on transcriptional activity were expected less probable. TLR3 promoter variants were not found to be associated with differences in TLR3 gene expression.
Fig. 1. TLR3 mRNA expression in PBMC and liver tissue samples from patients with chronic hepatitis C with regard to two TLR3 SNPs. Total cellular RNA from freshly isolated PBMCs (A) and liver tissue specimens (B) was analyzed for the expression of TLR3 mRNA in relation to GAPDH mRNA as a reference by commercially available real-time PCR gene expression assays as described in Materials and Methods Section. Data were related to the TLR3 promoter SNP rs5743305 under study and to the TLR3 exon 4 SNP rs3775291. Medians and P values are given. NS, non-significant.
with significant differences in PBMCs or in hepatic TLR3 mRNA expression (Fig. 1). Neither in healthy individuals, TLR3 promoter SNP genotype appeared to be related to the amount of TLR3 transcripts in PBMCs (data not shown).

In line with the lack of an association between TLR3 promoter genotypes and TLR3 gene expression, TLR3 promoter genotypes were not found to be significantly related to clinical data as serum transaminase activities (Table III) or histopathological manifestations (Table IV).

The polymorphism within TLR3 exon 4, which causes an amino acid exchange in the protein molecule, has been demonstrated to be functional in terms of mediating signaling [Ranjith-Kumar et al., 2007]. In chronic hepatitis C patients, this SNP was not found to be related to PBMCs but to hepatic TLR3 gene expression (Fig. 1). TT homozygous patients seemed to have higher hepatic TLR3 gene expression than C allele carriers ($P = 0.0191$). On the other hand, this SNP was not found to be associated with clinical data related to a chronic liver disease due to HCV infection: no significant relationship of TLR3 exon 4 variants was observed with serum transaminase activities (Table III) or with histological manifestations (Table IV).

An analysis of genotype distribution with regard to TLR3 promoter SNP revealed no significant difference among patients with HCV subtypes 1a, 1b, or 3a (Table V). In contrast, the TLR3 exon 4 genotype distribution with respect to HCV subtype, was not found to yield different distributions of TLR3 genotypes among patients with HCV subtypes 1a, 1b, or 3a infection. The proportion of patients with TT genotype and HCV subtype 1b infection amounts to 17.8%, only 9.1% of patients with HCV subtype 3a infection, and none of the patients with HCV subtype 1a infection were found to be homozygous for the variant allele T. A statistical comparison yielded a significant difference when patients with subtype 1a were compared to those with subtype 1b infection ($P = 0.0167$; Table VI).

**DISCUSSION**

Data revealed a lack of an association between hepatitis C patient’s TLR3 promoter genotype and TLR3 mRNA expression, which was assessed both in freshly prepared PBMC and liver tissue specimens. In line with these findings, no evidence was found for an association of TLR3 promoter genotypes and clinical parameters of the chronic disease.

The second SNP under study is located within the coding region of the TLR3 gene. The variant allele causes an amino acid exchange and the variant protein was shown to be impaired in its signaling function [Ranjith-Kumar et al., 2007]. This SNP was not found to be related to TLR3 gene expression in PBMCs, as expected, nor was it to clinical parameters of chronic HCV infection. However, a relationship with TLR3 mRNA expression in liver tissue in terms of slightly enhanced transcripts in patients who carry the TT

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**TABLE III. Biochemical Serum Parameters and the Number of Patients With Elevated Parameters in Chronic Hepatitis C With Regard to TLR3 rs5743305 and rs3775291 Genotype Distribution**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TLR3 rs5743305</th>
<th>TLR3 rs3775291</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT (n = 50)</td>
<td>TA (n = 62)</td>
</tr>
<tr>
<td></td>
<td>CC (n = 63)</td>
<td>CT (n = 58)</td>
</tr>
<tr>
<td>ASTa (mean ± SD)</td>
<td>44.2 ± 41.0</td>
<td>40.9 ± 44.5</td>
</tr>
<tr>
<td>ALTb (mean ± SD)</td>
<td>85.8 ± 102.6</td>
<td>78.5 ± 115.7</td>
</tr>
<tr>
<td>gGTc (mean ± SD)</td>
<td>44.3 ± 45.6</td>
<td>48.6 ± 62.5</td>
</tr>
<tr>
<td>No. of patients with elevated AST (%)</td>
<td>21 (42.0)</td>
<td>27 (43.5)</td>
</tr>
<tr>
<td>No. of patients with elevated ALT (%)</td>
<td>29 (58.0)</td>
<td>32 (51.6)</td>
</tr>
<tr>
<td>No. of patients with elevated gGT (%)</td>
<td>16 (32.0)</td>
<td>17 (27.4)</td>
</tr>
</tbody>
</table>

*P* values were calculated after stratifying for sex, age, and HCV subtypes. The number of patients with significantly elevated serum activities of transaminases (>2-fold of the upper normal limit) was considered.

aAST, aspartate aminotransferase. Upper normal limit is 19 U/ml for males, and 15 U/ml for females.
bALT, alanine aminotransferase. Upper normal limit is 23 U/ml for males, and 19 U/ml for females.
cgGT, γ-glutamyltransferase. Upper normal limit is 28 U/ml for males, and 18 U/ml for females.
genotype was found. Moreover, another association between TLR3 exon 4 SNP and the epidemiological parameter of HCV subtype infection became evident, in that patients who are homozygous for the wild-type allele or who are heterozygous are more or less equally infected by the predominant viral subtypes 1a, 1b, and 3a but none of the patients who are homozygous for the variant T allele were infected by HCV subtype 1a.

TLR3 is one of several PRRs sensing ss or ds viral RNA in man [Takeuchi and Akira, 2007]. Viral RNA is also recognized by TLR7, the expression of which is restricted to non-myeloid plasmacytoid DCs (pDCs), the main type I IFN producing cells that are activated in particular in response to systemic RNA virus infections [Takeuchi and Akira, 2007] and by cytosolic receptors in infected cells.

HCV has been demonstrated to target adapter molecules that link viral RNA sensing and downstream effector events as type I IFN induction [Gale and Foy, 2005]. HCV serine protease NS3/4A has been shown to induce proteolytic degradation of TRIF, MyD88, and interferon promoter stimulator-1 (IPS-1) leading to disruption of TLR3, MyD88-dependent TLRs, and retinoic acid inducible gene-1 (RIG-1) signaling in vitro [reviewed in Seki and Brenner, 2008]. All these interactions might be responsible for the absence of the activation of type I IFN genes by HCV in the human liver [Mihm et al., 2004] and might contribute to HCV persistence [Gale and Foy, 2005].

The lack of an association between the two TLR3 promoter and exon 4 variants and liver disease manifestations argues against a role of TLR3 in the outcome of liver disease due to chronic HCV infection. An involvement of TLR3 gene expression on mesangial renal cells, however, has been suggested for HCV-associated glomerulonephritis on the basis of the presence of immune complexes containing viral RNA, enhanced TLR3 mRNA expression, and an enhanced expression of chemokines [Wornle et al., 2006].

The finding of a different susceptibility to HCV subtype 1a infection in individuals who are homozygous for the variant T allele were infected by HCV subtype 1a.

### TABLE IV. Histological Manifestations in Chronic Hepatitis C Infected Patients With Regard to rs5743305 and rs3775291 Genotype Distribution

<table>
<thead>
<tr>
<th>Histological manifestations</th>
<th>rs5743305 genotype</th>
<th>rs3775291 genotype</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis activity</td>
<td>TT</td>
<td>TA</td>
<td>AA</td>
</tr>
<tr>
<td>Mild</td>
<td>30 (39.5)</td>
<td>34 (44.7)</td>
<td>12 (15.8)</td>
</tr>
<tr>
<td>Moderate or severe</td>
<td>20 (32.8)</td>
<td>28 (45.9)</td>
<td>13 (21.3)</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>34 (39.5)</td>
<td>37 (43.0)</td>
<td>15 (17.4)</td>
</tr>
<tr>
<td>Absent or mild</td>
<td>16 (31.4)</td>
<td>25 (49.0)</td>
<td>10 (19.6)</td>
</tr>
<tr>
<td>Moderate, marked, cirrhosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steatosis</td>
<td>38 (35.2)</td>
<td>50 (46.3)</td>
<td>20 (18.5)</td>
</tr>
<tr>
<td>Absent or mild</td>
<td>12 (41.4)</td>
<td>12 (41.4)</td>
<td>5 (17.2)</td>
</tr>
<tr>
<td>Portal lymphoid aggregates</td>
<td>30 (35.7)</td>
<td>43 (51.2)</td>
<td>11 (13.1)</td>
</tr>
<tr>
<td>Absent</td>
<td>20 (37.7)</td>
<td>19 (35.9)</td>
<td>14 (26.4)</td>
</tr>
<tr>
<td>Periportal lymphoid aggregates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>35 (39.3)</td>
<td>40 (44.9)</td>
<td>14 (15.7)</td>
</tr>
<tr>
<td>Bile duct damage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>15 (31.3)</td>
<td>22 (45.8)</td>
<td>11 (22.9)</td>
</tr>
</tbody>
</table>

The number of patients is followed by their percentage in brackets.

<sup>a</sup>P values were calculated after stratifying for sex, age, and HCV types.

### TABLE V. HCV Subtype Distribution in Chronic Hepatitis C Infected Patients With Regard to rs5743305 Genotypes

<table>
<thead>
<tr>
<th>HCV subtype</th>
<th>n</th>
<th>TT</th>
<th>TA</th>
<th>AA</th>
<th>MAF</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>31</td>
<td>16 (51.6)</td>
<td>12 (38.7)</td>
<td>3 (9.7)</td>
<td>0.290</td>
<td>0.1726&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1b</td>
<td>73</td>
<td>23 (31.5)</td>
<td>37 (50.7)</td>
<td>13 (17.8)</td>
<td>0.432</td>
<td>0.2466&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1a + 1b</td>
<td>6</td>
<td>1 (16.7)</td>
<td>3 (50.0)</td>
<td>2 (33.3)</td>
<td>0.3683&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.4031&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2a</td>
<td>1</td>
<td>0</td>
<td>1 (100.0)</td>
<td>0</td>
<td>0.386</td>
<td>0.3636&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2b</td>
<td>4</td>
<td>0</td>
<td>2 (50.0)</td>
<td>2 (50.0)</td>
<td>0.3636&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>22</td>
<td>10 (45.5)</td>
<td>7 (31.8)</td>
<td>5 (22.7)</td>
<td>0.386</td>
<td>0.4031&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

MAF, minor allele frequency.

<sup>a</sup>Cochran–Mantel Haenzel-test with age–sex-strata.

<sup>b</sup>Comparing HCV subtype 1a with 1b.

<sup>c</sup>Comparing HCV subtypes 1a, 1b, and 3a.

associated with the incidence of chronic HCV infection, severity, and with responsiveness to an IFN-α-based therapy [Schott et al., 2007, 2008]. Polymorphisms within the IRF-1 gene have been found to be related to resistance to HCV subtype 3a [Wietzke-Braun et al., 2006] or to human immunodeficiency virus-1 (HIV-1) infections [Ball et al., 2007]. In line with these findings, the two TLR3 SNP variants under investigation have been shown to be associated with low humoral and cellular immunity in response to measles vaccination [Dhiman et al., 2008]. Although animal models of virus infection argue against a role of TLR3 in the initial, cell-autonomous recognition of viral infection that induces a first wave of type I IFN production [reviewed in Akira et al., 2006], TLR3 has been shown to promote cross-priming of cytotoxic T lymphocytes against viruses that do not directly infect DCs after phagocytosis of infected material [Schulz et al., 2005].

Taken together, the recent observation of an association of TLR3 exon 4 genotype and HCV subtype infection supports the concept that genetic variations in viral RNA sensing molecules and/or molecules mediating type I IFN signaling do determine susceptibility to viral infections. A higher hepatic TLR3 expression might be related to the absence of HCV 1a infection in patients homozygous for TLR3 exon 4 variant T allele. Nevertheless, once a chronic infection is established, this polymorphism appears not to affect the clinical outcome of hepatitis C disease in general.

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