Value of $\alpha$-smooth muscle actin and glial fibrillary acidic protein in predicting early hepatic fibrosis in chronic hepatitis C virus infection

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Abstract

**Introduction:** $\alpha$-Smooth muscle actin ($\alpha$-SMA)-positive hepatic stellate cells (HSCs) are pericytes responsible for fibrosis in chronic liver injury. The glial fibrillary acidic protein (GFAP), commonly expressed by astrocytes in the central nervous system, is expressed in vivo in the liver in a subpopulation of quiescent stellate cells. The reports concerning GFAP expression in human liver are still conflicting. The aim of the study is investigation the utility of GFAP compared to $\alpha$-SMA as an indicator of early activated HSCs, in predicting fibrosis in chronic hepatitis C (CHC) patients.

**Material and methods:** With immunohistochemistry and a semi-quantitative scoring system, the expressions of $\alpha$-SMA and GFAP on HSCs in liver biopsies from patients with pure CHC ($n=34$), hepatitis C virus-induced cirrhosis ($n=24$), mixed CHC/schistosomiasis ($n=11$) and normal controls ($n=10$) were analysed.

**Results:** The immunoreactivity of $\alpha$-SMA and GFAP in perisinusoidal, periportal and pericentral areas was assessed. $\alpha$-Smooth muscle actin and GFAP-positive HSCs were significantly increased in all diseased groups compared with normal controls. In pure CHC with or without cirrhosis, perisinusoidal $\alpha$-SMA-positive HSCs were predominant in relation to GFAP-positive cells. On the other hand, GFAP-positive cells were predominant in the group of schistosomiasis as compared with the other diseased groups. It was noticed that expression of GFAP on perisinusoidal HSCs in CHC patients sequentially decreased with the progression of fibrosis.

**Conclusions:** Glial fibrillary acidic protein could represent a more useful marker than $\alpha$-SMA of early activation of HSCs in CHC patients and seems to be an early indicator of hepatic fibrogenesis.

**Key words:** $\alpha$-smooth muscle actin, glial fibrillary acidic protein, chronic hepatitis C, fibrosis, stellate cell.

Introduction

Hepatitis C virus (HCV) infection affects more than 170 million people worldwide, with the great majority (~85%) of patients developing chronic...
HCV infection [1]. Chronic hepatitis C (CHC) is considered the most common chronic liver disease (CLD) in Egypt, where prevalence of antibodies to HCV (anti-HCV) is approximately 10-fold greater than in the United States and Europe [2].

The main injury caused by HCV is hepatic fibrosis, as a result of a chronic inflammatory process in the liver. The development of CHC is better estimated by the fibrosis stage rather than by the necroinflammatory activity level [3]. Currently, the cirrhosis resulting from chronic virus C infection is the main cause of hepatic transplantation worldwide [4].

Schistosoma mansoni infects tens of millions of people in many developing countries [5]. In contrast to CHC infection, which causes severe necroinflammatory lesions and diffuse parenchymal damage, the schistosomal granulomatous inflammation is restricted to the periportal zone, does not induce hepatocellular degeneration or necrosis, and preserves hepatic lobular architecture and function [6].

Hepatic stellate cells (HSCs) exist in the space between parenchymal cells and sinusoidal endothelial cells of the hepatic lobule and store 80% of retinoids in the whole body. In pathological conditions such as liver fibrosis, HSCs lose retinoids, and synthesize a large amount of extracellular matrix (ECM) components. Morphology of these cells also changes from the star-shaped stellate cells to that of fibroblasts or myofibroblasts [7]. Indeed, the paradigm of stellate cell activation into contractile myofibroblasts as the major pathway in hepatic fibrogenesis associated with liver injury has dominated the focus of studies on this fascinating cell type [8]. In response to liver injury, human HSCs express α-smooth muscle actin (α-SMA), becoming "activated" and myofibroblast-like [9]. The correlation between HSC activation and necro-inflammatory activity and/or fibrosis stage is a point of much debate [10].

Glia fibrillary acidic protein (GFAP) is an intermediate filament protein that is found in glial cells [11]. Few studies have been performed in order to quantify the hepatic expression of GFAP at different stages of human chronic hepatitis [12]. The predominance of GFAP-positive cells observed in schistosomiasis suggests that HSCs have a major role in connective tissue deposition in the human schistosomal liver [13].

Hence, finding precocious markers of activated HSCs will be helpful in identifying early stages of hepatic fibrosis when the antiviral therapy is expected to reduce fibrosis progression. So, the aims of this work were: to correlate α-SMA and/or GFAP-positive HSCs with the severity of liver injury in the context of degree of necroinflammatory activity and stage of fibrosis in HCV infected patients, and to investigate the value of these markers as indicators of hepatic stellate cell activation, in predicting early fibrosis in CHC.

Material and methods

This prospective study was conducted on 69 patients with CLD admitted to the Department of Hepato-Gastroenterology, Theodor Bilharz Research Institute, Egypt in the period of 2004-2007. Fifty-eight were males (84.1%) and 11 were females (15.9%). Their ages ranged from 20 to 57 years with a mean of 40.82 ±8.98. All had circulating anti-HCV antibodies or HCV-RNA viraemia with no serological evidence of co-infection with hepatitis B virus. Patients with a history of antiviral therapy were excluded from the study. All patients were subjected to thorough clinical examination, routine laboratory investigations, abdominal ultrasound (Hitachi EuB-515A), rectal snip examination for detection of Schistosoma ova and upper endoscopy whenever indicated. Core liver biopsies were performed for all patients with safety precautions to minimize any consequent risk using a percutaneous ultrasound-guided Menghini needle for histopathological and immunohistochemical studies. The control group included 10 wedge liver biopsies taken during laparoscopic cholecystectomy from age- and sex-matched healthy subjects. They all had clinical, biochemical, serological, ultrasonographic and histological findings within the normal range. They were 8 males and 2 females. Their ages ranged between 26 and 52 years with a mean of 40.0 ±8.72 years. Our study followed the tenets of the Declaration of Helsinki [14].

Laboratory investigations

- Complete blood picture.
- Prothrombin time and concentration.
- Liver function tests including albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP).
- Hepatitis viral markers including hepatitis B surface antigen, anti-HBs antibodies, and total and IgM class antibodies against hepatitis B core antigen using enzyme immunoassay kits (Murex Diagnostics, Dartford, England). Anti-HCV antibodies using version V anti-HCV ELISA kit (Murex Diagnostics, Dartford, England). Circulating HCV-RNA was assayed to confirm the presence of HCV antigenemia by nested RT-PCR using a set of primers within the 5' non-translated region according to Saber et al. [15].
- Anti-nuclear antibodies (ANA) using ELISA kit (Quanta Lite™ ANA, Inova Diagnostics. Inc., USA) to exclude autoimmune hepatitis.

Histopathological studies

Serial sections (5-µm thick) from formalin-fixed, paraffin-embedded core liver biopsies were stained...
with haematoxylin and eosi n for routine histopathological examination. Masson trichrome stain was used for proper demonstration of fibrous tissue deposition. Liver sections with evidence of HCV infection were graded according to the degree of perivascular and intralobular inflammation, interface hepatitis and portal inflammation. The grades of CHC activity (scale A0-A3) and the stage of fibrosis (scale F0-F4) were assessed according to the METAVIR scoring system [16]. Grade 1 inflammation as well as the first and second stages of fibrosis were considered as a “low score”, while grades 2 and 3 inflammation as well as the third and fourth stages of fibrosis were considered as a “high score”. According to the above-mentioned clinical, serological and histopathological criteria, subjects included in this study were categorized into the following groups: (a) control (n = 10), (b) pure CHC with no cirrhosis or schistosomiasis (n = 34), (c) HCV-induced liver cirrhosis (LC) (n = 14) and (d) mixed lesion of CHC with schistosomiasis (n = 11).

Immunohistochemical technique

Five-µm thick sections from formalin-fixed, paraffin-embedded liver tissue from all cases were cut on microscopic slides coated with 3-amino propyl triethoxysilane (Sigma Chemicals; St. Louis, Missouri) for proper fixation of tissue sections on the slides and to minimize staining artefacts.

After deparaffinization and subsequent blockage of the endogenous peroxidase activity by incubation in 0.3% methanolic hydrogen peroxide (10 min), the sections were then washed in phosphate buffered saline (PBS). Antigen retrieval was performed by boiling the slides twice in 10 mmol/l citrate buffer solution (pH 6.0) (DAKO, Denmark) each for 5 min. Tissue sections were treated with normal horse serum (Dako, Denmark) for 10 min to avoid non-specific immunoreactivity. Duplicate liver sections were incubated overnight at 4°C with mouse monoclonal anti-α-SMA antibody (DAKO, clone 1A4) diluted 1:50 and ready-to-use mouse monoclonal anti-GFAP antibody (BioGenex, USA). Sections were then incubated at room temperature with biotinylated goat anti-mouse antibody for 10 min followed by streptavidin horseradish peroxidase conjugate (all from Dako, Denmark). The reaction was visualized by the addition of diaminobenzidine substrate solution (Dako, Denmark) followed by counterstaining with Mayer’s haematoxylin. Positive and negative control slides were included within each session.

The immunoexpression of both α-SMA and GFAP on HSCs was scored for perisinusoidal, perivascular and pericentral areas. Parenchymal HSCs were characterized by morphological criteria (perisinusoidally located, stellate-shaped cells residing in the parenchymal lobules or nodules) [12]. The number of positive and negative perisinusoidal HSCs was separately counted for each of α-SMA and GFAP under a light microscope at 200× magnification. For each slide, positive HSCs were counted in at least 7-10 microscopic fields of maximum staining intensity. The percentage of positive HSCs was calculated based on the total number of HSCs counted in each slide. The average percentage of positive HSCs was then calculated for each group [17]. The scores for perivascular and pericentral areas were determined semi-quantitatively as 0: no staining or < 3% of the region; 1) positive for 3-33% of the region, 2) positive for 34-66% of the region, and 3) positive for > 66% of the region [18].

Statistical analysis

Results are presented as the mean ± standard deviation. Statistical procedures were performed using SPSS statistical software (version 16, Chicago, Ill). Comparisons between multiple groups were carried out using one-way ANOVA and between two groups by Scheffé’s test. Where indicated, Pearson’s correlation coefficient was used. Probability values less than 0.05 were considered to be statistically significant.

Results

Sixty-nine patients suffering from chronic HCV infection were enrolled in this study. Past history of schistosomiasis was recorded in 11/69 (15.94%) of the studied patients and proved by rectal snip examination. The clinical and abdominal ultrasonographic features of the studied HCV patients are shown in Table I. While the majority of the patients (87%) presented with fatigue, none of them had clinically detected jaundice or ascites. Ultrasonographic examination revealed that none of the patients had a shrunken liver. As regards the sonographic texture of the liver, a coarse pattern was detected in 43.5%; however, a mixed bilharzial and bright pattern was detected in only 4.3% of the studied patients.

Immunohistochemical studies

The total number of HSCs immunostained by α-SMA and GFAP in each group was semi-quantitatively assessed in perisinusoidal, perivascular and pericentral areas of the hepatic lobules. Hepatic stellate cells were observed to be variable in size and shape, although most of them were found stretched with long cytoplasmic processes along the endothelial lining.

Immunorepression of α-SMA on hepatic stellate cells

In the control group, very few α-SMA-positive HSCs were detected only along the sinuoids
(Figure 1A) with a significant difference from the other groups ($p < 0.01$). In pure CHC patients, $\alpha$-SMA-positive cells were mainly located perisinusoidally and were more strongly and diffusely immunostained (Figure 1B). In the cirrhotic group, $\alpha$-SMA-positive HSCs were observed in the inter-parenchymal expanding septae as well as in the perisinusoidal spaces of the residual hepatic parenchyma, while in areas demonstrating regenerative activity, positive HSCs were confined to the periphery of the regenerative plates (Figure 1C).

The highest value of perisinusoidal $\alpha$-SMA-positive cells was encountered in pure CHC cases, with a significant difference compared with those mixed with schistosomiasis and controls ($p < 0.01$). However, the highest score of periportal and pericentral positive cells was detected in liver cirrhosis, with a significant difference from the other studied groups (Table I).

Concerning the grades of necroinflammatory activity and stages of fibrosis in CHC, patients with low grade (A0-A1) showed a significantly higher $\alpha$-SMA immunoreexpression on perisinusoidal HSCs when compared with those of higher grades (A2-A3) ($p < 0.01$). It was found that $\alpha$-SMA-positive HSCs were detectable in all stages of hepatic fibrosis. In cases of CHC with no evidence of fibrosis (F0), the mean value of perisinusoidal expression of $\alpha$-SMA-positive HSCs was $21.25 \pm 7.84$. However, the expression was completely absent in the periportal area, with a slightly higher value in the

| Characteristics | Patients (n = 69) |
|-----------------|------------------|
| Symptoms:       |                  |
| – Fatigue       | 60 (87%)         |
| – Rt. hypochondrial dull aching pain | 45 (65.2%) |
| Signs:          |                  |
| – Hepatomegaly  | 7 (10.15%)       |
| – Splenomegaly  | 0 (0%)           |
| Liver:          |                  |
| • Size:         |                  |
| – Average       | 55 (79.7%)       |
| – Enlarged       | 14 (20.3%)       |
| – Shrunken       | 0 (0%)           |
| • Texture:      |                  |
| – Normal        | 15 (21.7%)       |
| – Bright        | 21 (30.4%)       |
| – Coarse        | 30 (43.5%)       |
| – Mixed bilharzial and bright pattern | 3 (4.3%) |
| Spleen:         |                  |
| • Enlarged       | 10 (14.5%)       |
| Portal vein dilatation | 0 (0%) |

Table I. Clinical and abdominal ultrasonographic characteristics of the studied HCV patients
pericentral zone (0.63 ±0.49). In LC (F3-F4), perisinusoidal positivity was significantly decreased compared to the low stage (p < 0.01) and this relation was inverted in periportal and pericentral areas (Table III).

**Immunoeexpression of glial fibrillary acidic protein on hepatic stellate cells**

The control liver sections showed almost GFAP-negative HSCs with sporadic perisinusoidally positive cells found in the hepatic parenchyma (Figure 2A), with a significant difference vs. the other diseased groups (p < 0.01). In the CHC group, GFAP-positive HSCs were more evenly distributed throughout the hepatic lobule. However, in the cirrhotic group, GFAP-positive cells were not strongly and diffusely immunostained.

As patients with pure CHC showed a paucity of periportal and pericentral GFAP-positive HSCs, with a significant difference to the other diseased groups (p < 0.01), those mixed with schistosomiasis had the highest value of GFAP expression in the three zones (Table IV, Figure 2B). It was found that in low grade necroinflammatory activity (A0-A1), a significantly higher expression of GFAP-positive HSCs was detected in the perisinusoidal zone when compared with higher grades of activity (A2-A3) (p < 0.01). For stage F0, the expression of GFAP on perisinusoidal HSCs was the highest (24.38 ±5.77), with a significant difference vs. controls and patients with higher scores (p < 0.01) (Figures 2C, 2D). In contrast, patients with LC (F3-F4) showed the lowest value of GFAP expression on peri-
sinusoidal HSCs (9.83 ±5.01) and the highest value for periportal and pericentral HSCs, with a significant difference vs. the other cases with lower stages of fibrosis (Table V).

In CHC with or without LC, perisinusoidal α-SMA-positive HSCs were highly predominant in relation to GFAP-positive cells. Interestingly, the number of GFAP-positive cells was not only smaller in the former biopsies but the ratio to the α-SMA-positive cells was also inverted compared to those with mixed schistosomiasis (Figure 3).

Among the studied HCV-infected patients, there was no significant correlation between the expression of both markers and the clinico-laboratory variables of those patients, grades of necroinflammatory activity and stages of fibrosis. On the other hand, there was a significant correlation between the expression of both α-SMA and GFAP in liver biopsies of the studied HCV patients (r = 0.313, p < 0.01) (Figure 4).

**Discussion**

Hepatic fibrosis is a major feature of the liver injury that accompanies chronic HCV infection and in many patients leads to cirrhosis and end-stage liver disease. HSC activation, occurring in response to tissue injury and oxidative stress, is believed to be the central event in fibrosis [19]. However, the exact molecular events leading to HSC activation are not well understood. It is believed that stellate cells proliferate as a response to inflammation and secrete growth factors, cytokines, type I and type IV collagen, laminin and heparin sulphate. These cells
are known to contribute to the changes that take place in the sinusoidal structures during the early stage of hepatic fibrogenesis. The fibrillar extracellular matrix build-up in the space of Disse has been blamed for disrupting the circulation between hepatocytes and blood [20-22].

Although α-SMA positivity in a few stellate cells of the liver is normal, α-SMA expression is significantly increased in chronic hepatitis due to stellate cell activation. In other studies, stellate cell transcription has been claimed to be controlled by α-SMA [23-26]. In the present study, significant numbers of α-SMA-positive HSCs were detected throughout the examined liver sections in patients with chronic HCV infection, whereas in normal controls α-SMA-positive cells were poorly detected.

Table IV. Immunoexpression of GFAP on HSCs in the studied groups

| Groups                               | Perisinusoidal, mean ± SD | Periportal, mean ± SD | Pericentral, mean ± SD |
|--------------------------------------|---------------------------|-----------------------|------------------------|
| Control (n = 10)                     | 2.3 ±1.89                 | 0.00                  | 0.00                   |
| Pure chronic hepatitis C (n = 34)    | 14.5 ±3.69<sup>a</sup>    | 0.70 ±1.06            | 0.90 ±0.99             |
| Liver cirrhosis (n = 24)             | 9.83 ±5.01<sup>b</sup>    | 2.13 ±0.95<sup>b</sup>| 1.88 ±0.95<sup>b</sup> |
| Mixed CHC/schistosomiasis (n = 11)   | 20.46 ±15.57<sup>a,c</sup>| 2.36 ±0.81<sup>b</sup>| 2.36 ±0.81<sup>b</sup>|

*Percentage of positive cells/group, *semi-quantitative scoring of positive cells/group, *p < 0.01 vs. control, *p < 0.01 vs. pure CHC (chronic hepatitis C), *p < 0.05 vs. liver cirrhosis
In contrast, their prevalence was not associated with the severity of fibrosis. These results are quite consistent with those obtained by Martinelli et al. and Carotti et al. [17, 26]. Because α-SMA is a specific marker for smooth muscle cell differentiation [27], it has been used to identify activated HSCs that show a myofibroblastic phenotype [28, 29]. However, the association between the α-SMA-positive HSCs and the extent of fibrosis is controversial. A lack of correlation between the prevalence of α-SMA-positive HSCs and fibrosis severity in chronic liver disease was reported by Levy et al. [30], whereas a positive correlation has been found in other studies [10, 26, 31]. The precise subpopulation of α-SMA-positive HSCs/myofibroblasts related to fibrosis is still debated [32, 33].

The presence of α-SMA-positive HSCs in stage 0 fibrosis CHC disease suggested that HSCs were already activated by the virus infection, even in the absence of detectable fibrosis. These cells may return to an inactive state once fibrosis is well established, especially in the absence of ongoing hepatic inflammation. In the present study, the mean value of perisinusoidal HSCs positive for α-SMA in CHC cases with stage 0 fibrosis was 21.25 ±7.84 and in advanced stage 3-4 fibrosis was 16.5 ±7.41, which were closely related and distinct from those in stage 1-2 fibrosis: 28.24 ±16.12. In CHC, both Th1 and Th2 subsets of lymphocytes are important in regulating host responses via cytokine production [34, 35]. In general, Th1 cells produce cytokines such as interferon-γ and tumour necrosis factor that promote inflammation and cell-mediated immunity in an attempt to control infection. Th2 lymphocytes produce cytokines, especially interleukin 4, that favour fibrogenesis in liver injury to a greater extent than Th1 lymphocytes [36-38]. The increased abundance of IL-4 message identified in stage 1-2 fibrosis is therefore likely to contribute to the overall fibrogenic process. The characterization of a more specific marker for activated HSCs could be helpful in improving the predictive role of HSC detection.

### Table V. GFAP immunoexpression on HSCs in controls as well as HCV patients with different grades and stages

| Groups                      | Perisinusoidal*, mean ± SD | Periportal*, mean ± SD | Pericentral*, mean ± SD |
|-----------------------------|-----------------------------|------------------------|-------------------------|
| Control (n = 10)            | 2.3 ±1.89                   | 0                      | 0                       |
| Chronic hepatitis C (n = 69)| 16.10 ±8.38*               | 1.09 ±1.19             | 1.17 ±1.01              |
| – Low grade (A0-A1) (n = 39)| 21.21 ±6.91*               | 0.46 ±0.94*            | 0.77 ±0.9*              |
| – High grade (A2-A3) (n = 30)| 9.47 ±4.64*               | 1.9 ±0.96              | 1.7 ±0.92               |
| – F0 (n = 24)               | 24.38 ±5.77*               | 0.13 ±0.34             | 0.38 ±0.49              |
| – Low stage (F1-F2) (n = 21)| 13.81 ±6.26*               | 1.0 ±1.1*              | 1.29 ±0.9*              |
| – High stage (F3-F4) (n = 24)| 9.83 ±5.01*               | 2.13 ±0.95*            | 1.88 ±0.95*             |

*Percentage of positive cells/group, *semi-quantitative scoring of positive cells/group, A – necroinflammatory activity, F – stage of fibrosis, *p < 0.01 vs. control, †p < 0.01 vs. high grade, ‡p < 0.01 vs. F0, ††p < 0.05, ‡‡p < 0.01 vs. high stage
Gliai fibrillary acidic protein is an intermediate filament first identified and characterized in astroglial cells [39]. Hepatic stellate cells share several features with astrocytes of the central nervous system, such as juxtaposition to the capillaries, a stella shaped appearance [40], and response to tissue injuries [41].

The role of GFAP expression in HSCs is currently unknown. Previous studies in rodents showed that HSCs contained an unusually broad spectrum of intermediate filament proteins [42, 43]. The accumulation of GFAP/desmin-positive HSCs in an early stage of fibrosis [44], the proliferation of cells [45], and the expression of extracellular matrix genes and proteins [46, 47], which are hallmarks of activated HSCs, suggested that an increased expression level of GFAP by HSCs could be related to their initial activation changes.

Reports concerning GFAP expression in human liver are conflicting. A rim of GFAP-positive cells around portal tracts in normal liver and increased staining in the cirrhotic nodule without staining in the fibrous septa have been described [48]. In another study [49], GFAP was not detected in normal liver HSCs but was detected in focal periporal areas in cirrhotic liver. Few studies have been performed in order to quantify the hepatic expression of GFAP at different stages of human chronic hepatitis [12]. In the present study, GFAP-expressing HSCs seem to be related to early phases of fibrotic tissue deposition. We found that the percentage of perisinusaloid GFAP-positive HSCs was lowest in the cirrhotic patients compared to those with lower stages of fibrosis, especially stage 0 fibrosis; this means that the extent of GFAP expression correlates negatively with the stage of fibrosis. This might have a great impact on selection of patients for antiviral treatment. Currently, patients with low grade necroinflammation and stage 0 fibrosis are not treated but are followed up with the risk of decreased response to treatment if fibrosis later ensues. In view of our results, those patients can be candidates for treatment if the follow-up liver biopsy showed an appreciable decrease in the expression of GFAP on HSCs even without aberrant histopathological changes in grading or staging.

In the present study, we observed a remarkably higher number of α-SMA and GFAP-positive cells in patients with mixed CHC/schistosomiasis compared with the normal controls. Moreover, we found a significant predominance of GFAP-positive HSCs in relation to α-SMA-positive ones, suggesting that HSCs and not liver myofibroblasts may be the main cellular source of connective extracellular matrix deposited in portal spaces of schistosomiasis. These data agree with Chang et al. [13] and the experimental studies which found that activated Ito cells participate in the deposition of extracellular matrix in periportal schistosomal granulomas [50, 51].

In this study, α-SMA expression correlated significantly with the expression of GFAP in chronic HCV-infected patients. Carotti et al. [17] found that α-SMA and GFAP immunoreactivity partially overlapped, some HSCs being α-SMA-positive and GFAP-positive; others are stained either for α-SMA or GFAP.

In conclusion, GFAP could represent a useful marker of early activation of HSCs in chronic HCV disease. GFAP and α-SMA dependent activation of HSCs precedes fibrotic tissue deposition, inversely correlating with fibrosis and necroinflammatory activity. This might have a great impact on selection of patients for antiviral treatment. The GFAP-positive cells could be the precursors of fully activated HSCs identified by α-SMA immunoreactivity, or they could represent a subpopulation of different origin. Further studies are needed to investigate the origin of GFAP-positive HSCs in human liver.

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