Immunohistochemical detection of C-100 hepatitis C virus antigen in formaldehyde-fixed paraffin-embedded liver tissue. Correlation with serum, tissue and *in situ* RT-PCR results

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We localized HCV C-100 protein in liver biopsies of 15 patients with chronic hepatitis C using immunohistochemistry. The results were compared to serum, tissue extract analysis of HCV RNA and *in situ* RT-PCR described in a previous study. HCV was detected in 80% of the sera tested, in 40% of the tissue extracts and in 80% and 60% of the tissue sections tested by immunohistochemistry and *in situ* RT-PCR respectively. Compared to the serum positive cases, 83% and 67% of the cases were respectively positive with immunohistochemistry and *in situ* RT-PCR and 41% were positive with tissue extract detection. Compared to the tissue extract positive cases, 25% and 50% of the cases were respectively positive with immunohistochemistry and *in situ* RT-PCR. Finally, 75% of the cases positive by immunohistochemistry were also positive by *in situ* RT-PCR. These results underline the complementarity of the different methods for the precise diagnosis of hepatitis C.

Key words: chronic hepatitis C, immunohistochemistry, C-100 protein, hepatitis C virus RNA, *in situ* RT-PCR.

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Several studies based on the detection of hepatitis C virus (HCV) RNA or virus-related proteins in formaldehyde-fixed paraffin-embedded liver biopsies have been already reported. These studies used reverse transcriptase-polymerase chain reaction (RT-PCR) performed on extracted RNA (Guerrero et al., 1997; Svoboda-Newman et al., 1997; Dries et al., 1999; Soguero et al., 2000), *in situ* hybridization (Haruna et al., 1993; Nouri-Aria et al., 1993; Tang et al., 1995; Cho et al., 1996; Felgar et al., 1996; Kojima et al., 1996; Sansonno et al., 1996; Sansonno et al., 1997; Ohishi et al., 1999; Chang et al., 2000; Rodriguez-Inigo et al., 2000), *in situ* RT-PCR (Lidonnici et al., 1995; Nuovo, 1998; Walker et al., 1998; Biagini et al., 2001) and immunohistochemistry (IMH) (Gonzalez-Peralta et al., 1994; Komminoth et al., 1994; Chamlian et al., 1996; Vartanian et al., 1996; Kawamura et al., 1997; Moriya et al., 1997; Muramarsu et al., 1997; Brody et al., 1998; Yasui et al., 1998; Errington et al., 1999; Gomez et al., 1999; Kim et al., 1999; Nakopoulo et al., 1999; Nayak and Sathar, 1999; Wölk et al., 2000). Analysis of the corresponding data concerning the intracellular localization of HCV RNA and proteins revealed controversial results. Most studies located HCV constituents in cytoplasm of hepatocytes (Gonzalez-Peralta et al., 1994; Komminoth et al., 1994; Tang et al., 1995; Sansonno et al., 1996; Vartanian et al., 1996; Sansonno et al., 1997; Bettinger et al., 1999; Nakopoulo et al., 1999; Ohishi et al., 1999; Chang et al., 2000; Rodriguez-Inigo et al., 2000; Wölk et al., 2000) whereas some other works described a cytoplasmic and/or nuclear localization (Haruna et al., 1993; Nouri-Aria et al., 1993; Lidonnici et al., 1995; Chamlian et al., 1996; Cho et al., 1996; Felgar et al., 1996; Kawamura et al., 1997; Morimoto et al., 1997; Moriya et al., 1997; Brody et al., 1998; Walker et al., 1998; Errington et al., 1999; Kim et al., 1999; Nayak and

Table 1. Comparison of immunohistochemical results, HCV-RNA detection by RT-PCR, histological parameters and biological markers.

Cases	Knodell score[1]	ALT[2] (IU/L)[1]	HCV serology[1]	Seric HCV RNA[1]	Liver tissue extract[1]	Immunohistochemistry		
						In situ staining[1,3]	Nuclear Staining[3]	Cytoplasmic staining[3]
1	10	12	+	+	+	0	0	0
2	8	12	+	+	–	3	0	3
3	5	15	+	+	+	1	0	3
4	7	150	+	+	+	0	0	0
5	13	26	+	+	+	3	4	0
6	10	36	+	+	–	0	0	4
7	5	22	+	–	–	0	1	4
8	11	18	+	+	–	2	0	4
9	14	25	+	+	–	2	1	0
10	10	22	+	+	–	2	2	0
11	5	16	+	–	+	0	0	0
12	13	11	+	–	–	1	1	0
13	12	14	+	+	+	2	0	4
14	5	11	+	+	–	0	0	3
15	5	18	+	+	–	3	3	0

[1]according to Biagini et al., (2001); [2]ALT: alanine transaminase; [3]the number of stained cells was expressed semi-quantitatively as 0: no staining, 1: 1 to 10% of the stained cells, 2: 10 to 30%, 3: 30 to 40% and 4: more than 40%.

Sathar, 1999). Furthermore, no significant correlation between diagnosis, *in situ* detection of viral RNA and immunohistochemical results has been frequently described (Kojima et al., 1996; Vartanian et al., 1996; Negro and Hadengue, 1997; Scheuer et al., 1997; Dries et al., 1999; Gomez et al., 1999; Biagini et al., 2001). In addition, conflicting results on the correlation between serum and hepatic HCV RNA detected in tissue extracts or *in situ* RT-PCR and IMH have been underlined (Gonzalez-Peralta et al., 1994; Lidonnici et al., 1995; Gomez et al., 1999). Different sensitivities in detecting HCV in paraffin-embedded and in formaldehyde-fixed tissues have been described by several authors (Blight et al., 1992; Tsutsumi et al., 1994; Guerrero et al., 1997). This could be not only due to the fixation processes but also to some denaturation occuring during storage of liver biopsies (Akyol et al., 1992) and/or to the low level of HCV components and their heterogeneous distribution in liver tissue.

On the other hand, controversial results concerning the specificity of immunohistochemical methods applied on paraffin sections of formaldehyde-fixed liver biopsies have been related. Some authors described unreliable immunohistochemical results (Komminoth et al., 1994; Vartanian et al., 1996; Svoboda-Newman et al., 1997; Gomez et al., 1999), while others confirmed the specificity and sensitivity of their immunohistochemical methods for detection of HCV in fixed liver tissue (Brody et al., 1998).

Therefore, the aim of this study was first to perform HCV immunohistochemical detection in patients with histologic diagnosis of chronic hepatitis C, and subsequently to compare these results to previous findings related to HCV RNA detection approaches (Biagini et al., 2001).

Materials and Methods

Materials

15 cases of chronic hepatitis C positive for HCV serology (third generation Elisa tests) studied in a previous work (Biagini et al., 2001) were selected (Table 1). Liver biopsies were fixed for 4 h in 4% buffered formalin solution, routinely embedded in paraffin and cut into 5 µm serial sections.

Immunohistochemistry

A monoclonal antibody anti-C100-3 non-structural protein (clone TORDJI 22, Clonatec-Biosoft, France) was used and two immunohistochemical detection methods were tested: alkaline phosphatase (AP) detection (EnVision kit from Dako, France) and immunogold silver staining (IGSS), which was previously described (Chamlian et al., 1996).

The list of reagents and sources used in this study is given in Table 2. Sections were treated with primary antibody 1:100 diluted in antibody diluent (Dako), during 50 min at room temperature (AP method) or during 2 h at 37°C (IGSS method), with secondary antibody for 10 min at room temperature

Table 2. Reagents and sources.

Procedure	*Primary Antibody*	*Secondary Antibody*	*Staining Technique*
AP*	Mouse monoclonal IgG1 to HCV C100-3, clone TORDJI-22 (217-91, Clonatec-Biosoft, France)	Alkaline phosphatase labelled polymer conjugated to goat anti-mouse IgG (EnVision system, K 1396, Dako, France)	Fast red, levamisole (EnVision system, K 1396, Dako, France)
IGSS**	Mouse monoclonal IgG1 to HCV C100-3, clone TORDJI-22 (217-91, Clonatec-Biosoft, France)	Goat gold-labelled antimousse IgG (RPN 451, Auroprobe, LM, Amersham Biosciences, France)	Silver enhancement (RPN 491, intenSE M, Amersham Biosciences, France)

**AP. alkaline phosphatase; **IGSS: immunogold silver staining.*

(AP method) or 1:40 diluted in Dako antibody diluent during 1 h at room temperature (IGSS method). The final staining procedure was Fast Red with levamisole for 4 min at room temperature (AP method) and silver enhancement for 14 min at 23°C (IGSS method). Slides were washed in PBS or TBS (AP procedure) and in distilled water after this final step and counterstained in Mayer's hemalum solution. They were then mounted in aqueous permanent mounting medium after AP procedure or dehydrated and mounted in synthetic resin (IGSS method).

Two controls were performed:

(i) Omission of the first step of immunohistochemical procedures (incubation without primary antibody).

(ii) Use of a non-immune mouse serum 1:100 diluted in Dako antibody diluent instead of primary monoclonal antibodies.

The number of stained cells was expressed semi-quantitatively as 0: no staining, 1: 1 to 10% of the stained cells, 2: 10 to 30%, 3: 30 to 40% and 4: more than 40%.

Statistical analysis

Statistical analyses were performed using the Statcalc module of the Epi Info program (Version 6, CDC, Atlanta GA). Frequency distributions were compared using the chi-square (χ^2) test (Mantel-Haenszel and Yates analysis) and the Fischer exact test.

The relationships between immunohistochemical staining and *in situ* HCV RNA detection, biological markers (serum and tissue extract HCV RNA status, alanine amino transferase levels), and clinical data (Knodell score) were analyzed.

Results

Table 1 summarized immunohistochemical results and previous data on HCV RNA detection by RT-PCR in sera, tissue extract and by *in situ* RT-PCR.

Immunohistochemistry

The C100-3 antigen was mainly located in hepatocytes (Table 1). With AP detection method, nuclei were stained (Figure 1a) and coarse clumped granular staining was found in cytoplasm of some hepatocytes (Figure 1b). The stained cells were isolated or clustered. To a lesser extend, nuclei of Kupffer, bile duct and mononuclear cells were also stained. The staining pattern was similar with IGSS method. However, IGSS gave a stronger reaction than AP as shown in Figure 1d,e. No staining was found in control slides (Figure 1c, f).

Nuclear and/or cytoplasmic labeling was found in 80% of the cases. Among them, 6 cases showed a cytoplasmic staining and 5 a nuclear one. Only one case (case 7) exhibited both cytoplasmic and nuclear reactions. 75% of the cases positive by IMH were also positive by *in situ* RT-PCR detection. In 20% of the cases, both IMH and *in situ* RT-PCR showed negative results. In addition, 3 cases were only detectable by IMH. Finally, compared to the cases positive by RT-PCR in serum and to the cases positive by RT-PCR on tissue extracts, 83% and 25% of the cases were respectively positive using IMH.

Statistical analysis

Statistical analysis did not show significant relationship between immunohistochemical or *in situ* RT-PCR stainings and serum, tissue extract HCV

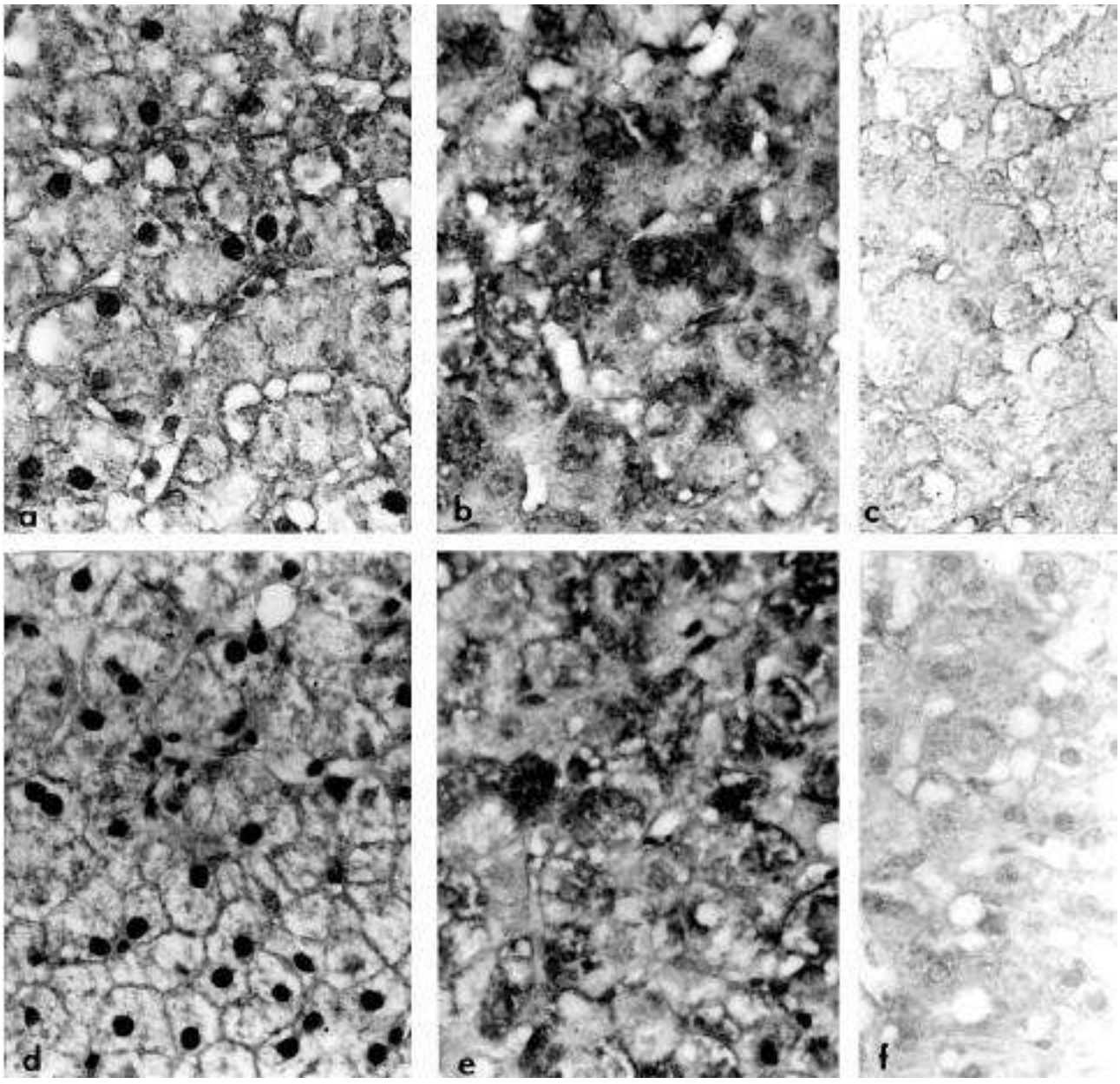

Figure 1. Liver sections from patients with chronic hepatitis C. Immunohistochemical staining of HCV C100-3 non-structural protein (magnification, x450). (a-c) Alkaline phosphatase detection (AP). (a) nuclear staining in a cluster of hepatocytes. (b) Granular cytoplasmic staining in some hepatocytes. (c) No staining in control section using non-immune mouse serum as primary antibodies. (d-f) Immunogold silver staining (IGSS). (d) Strong nuclear staining in hepatocytes. (e) Granular cytoplasmic staining in clustered hepatocytes. (f) No staining in control section using non-immune mouse serum as primary antibodies.

RNA status (positive/negative), ALT levels and Knodell scores.

Discussion

In a previous paper, (Biagini et al., 2001), we emphasize the lack of clear correlation between HCV RNA molecular detection methods in biologic samples of patients with chronic hepatitis C, thus confirming the literature data (Akyol et al., 1992; Bresters et al., 1994; Negro and Hadengue, 1997; Scheuer et al., 1997; Dries et al., 1999; Chang et al., 2000). Moreover, our findings based on *in situ* RT-PCR approach revealed a nuclear detection of HCV RNA as previously described by several authors (Haruna et al., 1993; Nouri-Aria et al., 1993; Cho et al., 1996; Walker et al., 1998), but in discrepancy with others studies showing a cytoplas-

mic localization (Tanaka et al., 1993; Tang et al., 1995; Kojima et al., 1996; Lau et al., 1996; Sansonno et al., 1996; Sansonno et al., 1997; Bettinger et al., 1999; Ohishi et al., 1999; Chang et al., 2000; Rodriguez-Inigo et al., 2000).

In this study, the immunolocalization of the C100-3 non-structural protein of HCV was mainly found in cytoplasm and nuclei of hepatocytes. In addition, in most of the cases, hepatocytes with cytoplasmic staining were devoid of nuclear staining and inversely. These different localizations were also described in the literature which attributed cytoplasmic or nuclear, or cytoplasmic and nuclear localization to HCV structural and non-structural proteins (Gonzalez-Peralta et al., 1994; ; Komminoth et al., 1994; Vartanian et al., 1996; Ishido et al., 1997; Kawamura et al., 1997; Moriya et al., 1997; Muramatsu et al., 1997; Brody et al., 1998; Yasui et al., 1998; Errington et al., 1999; Gomez et al., 1999; Honda et al., 1999; Kim et al., 1999; Nakopoulo et al., 1999; Nayak and Sathar, 1999; Wölk et al., 2000).

Immunohistochemical results showed here a good correlation between *in situ* RT-PCR method and IMH (75% of cases *in situ* RT-PCR positive were also positive with IMH). Moreover, three cases negative by the *in situ* RT-PCR approach were positive with immunohistochemical detection. These results indicate that IMH seems a sensitive method to detect HCV using the TORDJI-22 monoclonal antibody. Finally, statistical analysis did not show significant relationship between the different biological parameters, in particular between high or low Knodell scores and *in situ* RT-PCR staining (Biagini et al., 2001) or immunodetection.

In conclusion, our results show that the methods described here are complementary for HCV detection. The presence of HCV protein in the nuclei of hepatocytes in patients with chronic hepatitis C may play a role in the persistence of HCV infection (Errington et al., 1999), whereas the detection of HCV RNA in hepatocytes nuclei could also suggest that HCV, as other members of the *Flaviviridae* family, may have a nuclear replicative phase (Westaway, 1987).

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