LETTER TO THE EDITOR

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Fluorescent RT *in situ* PCR detection of *MRP1* mRNA in human HCV infected liver

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Chronic hepatitis C is now a major cause of chronic liver disease, cirrhosis and hepatocellular carcinoma. Approximately 20% of cases of acute viral hepatitis are due to hepatitis C. Cirrhosis develops in more than 25% of patients with chronic infection and each year hepatocellular carcinoma occurs in 1-3% of patients with cirrhosis due to HCV (Hoofnagle 1999).

Patients with hepatocellular carcinoma are characterized by nonresponsiveness to chemotherapeutic agents. A cause of refractivity to treatment has been ascribed to the overexpression of the Pgp (MDR) protein (Kim *et al.* 1999), and the additional involvement of multidrug resistance-associated proteins (MRPs) has been also hypothesized (Minemura *et al.* 1999).

Recently, a possible relationship between HBV and the drug sensitivity phenotype of cancer cells has been hypothesized, suggesting that a transcription transactivator encoded by hepatitis B virus X gene can transactivate the *MDR1* gene (Dunn *et al.* 1996). Until now, no data have been reported on a possible correlation between HCV infection and multidrug resistant phenotype during chronic infection or in hepatocarcinoma due to hepatitis C.

The multidrug resistance-related protein MRP1, together with MDR1, is the major mechanism of resistance to chemotherapeutic agents in different tumors (Hipfner *et al.* 1999). Since its presence is very low in normal liver, but an overexpression has been reported in hepatocarcinomas as well as during liver regeneration (Matsunaga *et al.* 1998), we considered the hypothesis of a *MRP1* varied activation during HCV infection. Interest in this issue is also justified by the recent *in vitro* studies of Takeuci *et al.* (1999) which indicate that *MRP1* expression in hepatocytes is reduced by interferon alfa, the only efficient agent in the therapy of chronic hepatitis C.

Different technical approaches have been used for the evaluation of *MRP1* expression, from immunohistochemistry and *in situ* hybridization to Northern blot analyses and RT-PCR (Legrand *et al.* 1996).

In this study we applied a rapid, sensitive and robust *MRP1* expression detection protocol based on direct RT-IS-PCR technology and fluorochrome-modified ($dCTP^{Cy3}$) nucleotides in order to test the presence of *MRP1* mRNA in liver biopsies of individuals with severe HCV infection.

Paraffin-embedded liver biopsies from ten patients with severe HCV infection (coming from the Istituto di Anatomia Patologica, University of Napoli), previously fixed with 10% buffered formalin for 16 hours, were dewaxed by xylene treatment (1 hour at room temperature) and rehydrated

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to nuclease-free water through graded fresh acqueous solutions of ethanol (100%, 95%, 70%). Slides were rinsed in PBS for 2 min, immersed in a 0.01% Triton-X 100/PBS solution for 2 min, rinsed in PBS for 2 min. The permeabilization of liver biopsies was performed using 2 μg/ml of proteinase K (Boehringer Mannheim) at 37°C for 30 min.

After proteinase K inactivation by heat (95°C for 1 min) the biopsies were treated with RNA free DNAase (Celbio, Milan) (50 U) at 37°C overnight. Reverse transcription (RT) mix, containing random hexamers, were placed on HCV-infected liver biopsies and the reaction mix was covered with silicon AmpliCover Discs (PE Biosystems) and assembly completed with overlay of the AmpliCover Clips (PE Biosystems). RT reaction was performed with MMULV reverse transcriptase (PE Biosystems) for 30 min at 42°C in the GeneAmp *in situ* PCR System 1000 (PE Biosystems).

Slides were then disassembled and the biopsies post-fixed by heat treatment (60°C for 5 min). PCR amplification of the cDNA was carried out using the GeneAmp *in situ* PCR System 1000 (PE Biosystems). The direct fluorescent *in situ* PCR was performed as follows: slides were heated at 70°C before starting the reaction ("hot start"); the *in situ* PCR solution (preheated at 70°C) was 10 mM Tris, 50 mM KCl, pH 8.3, dTTP, dCTP, dGTP, 200 μM each, 200 μM dATP and 5 μM FITC-dATP (Boehringer Mannheim), *MRP1* specific primers (forward 5'-CTGTTTTGTTTTCGGGTTCC-3'; reverse 5'-GATGGTGGACTGGATGAGGT-3'), 0.5 μM each, and 10 units for reaction of IS-Amplitaq (PE Biosystems). The following thermal cycle was used: denaturation at 94°C for 1 min, annealing at 58°C for 30 sec and extension at 72°C for 2 min, repeated 15 times. After the amplification, the slides were washed twice with PBS for 5 min and then counterstained with Vectashield-DAPI (Vector Labs, Burlingame, CA) and directly analyzed under a fluorescent microscope (Leica Microsystem, Heerbrugg CH).

Controls were used for RT and IS-PCR, without RT and without primers. Primers were excluded from the PCR mix to establish whether non-specific elongation by Taq polymerase had occurred because of the nicks in the fixed cDNA. A non-infected liver biopsy from healthy subject was used as "no target control".

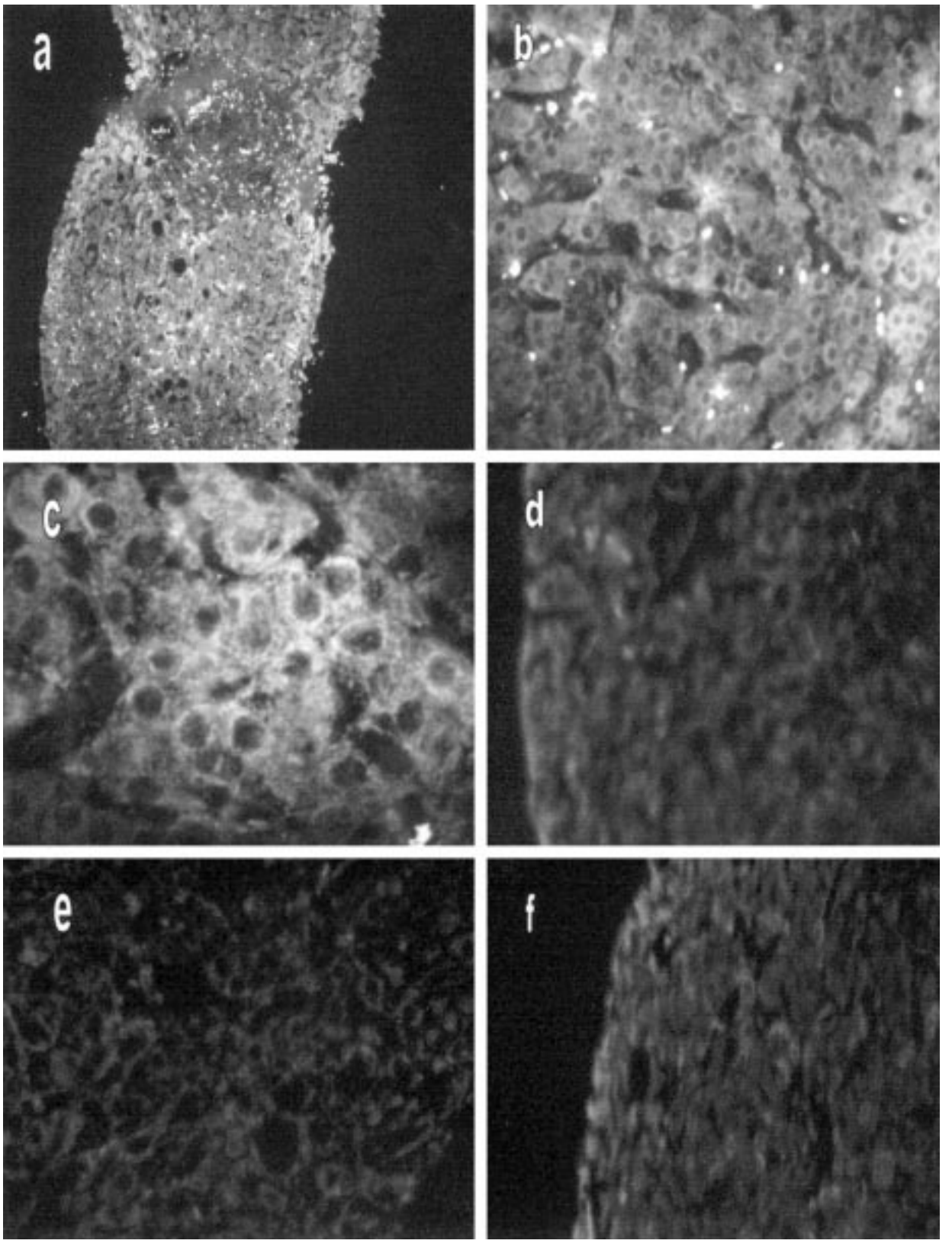
The morphology of HCV infected liver biopsies was maintained despite the multiple steps of fixation, permeabilization and RT, PCR (with thermal cycling).

Figure 1 shows the results of direct fluorescent RT-IS-PCR on HCV biopsies. We examined ten liver biopsies from patients with severe chronic hepatitis C, graded according to the severity of the necroinflammatory process (diffuse hepatocellular dammage). A strong positive signal for *MRP1* was detected in all specimens (Figure 1a, 1b,1c), indicating a significant expression in the infected liver tissues. In contrast with the nearly absent expression reported in normal liver (Figure 1f), a consistent presence of MRP1 in HCV infected liver may support the hypothesis of an involvement of MRP1 in inflammatory conditions and in cellular dammage (Hipfner *et al.* 1999). Figure 1d shows the results obtained with the negative control, in which the RT reaction was omitted: no positive signal of *MRP1* expression was found and the level of background was very low.

A negative result was also obtained for the no primer control (Figure 1e). False positive signals, due to the Taq polymerase gap filling and nick repair activity on damaged nuclear DNA, were not detected because of the DNAase (RNA free) overnight pre-treatment previous to the RT and amplification steps.

We have described a new sensitive, specific and reproducible method to detect *in situ MRP1* expression that could be suitable for liver biopsies as well as for different tissue specimens. The possibility to detect varied expression of *MRP1* in liver pathologies other than cancer will add important clues on the role of the encoded protein and its regulation.

Fig. 1 - (Panel a, b, c) RT fluorescent *in situ* PCR on paraffin embedded liver biopsies from patients with severe HCV infection. A positive signal of amplification is clearly detectable, *MRP-1* expression was visible after 15 cycles. Magnification 100 (a), 400 (b), 1000 (c). **(Panel d)** No RT control. RT reaction has been omitted and no positive signal of *MRP1* expression has been found in liver biopsies. Magnification 200. (**Panel e)** No primer control. No false positive signals due to the Taq polymerase gap filling and nick repair activity were detected. Magnification 200. **(Panel f)** No target control. No signal of amplification has been found in a biopsy from the liver of a healthy control. Magnification 200.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