Preface to the Second Edition

The first edition of this book, published in 1998, presented a large collection of authoritative protocols from the first decade of HCV research, outlining clinical diagnostics, genotyping, and molecular biology of the virus. One of the largest obstacles to productive research on the HCV life cycle had been the lack of a robust cell-culturing system capable of HCV replication and infection. We now possess such systems, and as a result, the past decade has witnessed an explosion of innovative research directed at understanding HCV biology and, more specifically, RNA replication in cell-culture replicon models. More recent advances in the production of infectious particles and infection of cultured hepatoma cells as well as chimpanzees will surely open a new chapter of studies examining the full life cycle of the virus by means of the new infection model. This advance is especially important for traditional virologists and immunologists, as they are now able to study viral assembly, entry, and pathogenesis as well as the innate and adapted immunity of the host. In other words, we have finally reached a point where virtually all the important questions regarding this human pathogen and its interaction with the host can be addressed in a laboratory setting.

This new edition of *Hepatitis C* strives to reflect this major thrust in the basic research of HCV and to provide a compilation of cutting-edge research techniques that are currently used by HCV labs worldwide to study HCV infection and replication in vitro. In addition, in keeping with the style of the first edition, the book opens with updated chapters discussing important methods of clinical detection and quantification of HCV infection. These are followed by several chapters detailing current systems for classifying HCV genotypes, numbering HCV sequences, and determining quasispecies. Finally, the volume concludes with protocols for the characterization of immune responses to HCV and a unique chapter describing mathematical modeling of HCV RNA kinetics. We hope that both basic scientists and clinical researchers will find this book useful as a reference guide to lab research using HCV as a model.

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Chapter 3

Immunohistochemical Detection of HCV Proteins in Liver Tissue

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Abstract

Detection and localization of HCV in liver tissue are vital for diagnostic purposes and clinical management of HCV-infected patients, as well as for the elucidation of viropathological mechanisms. The fragility of HCV RNA and the low levels of viral expression in infected tissues are a constant limitation in molecular assays for HCV characterization. HCV antigen detection, by immunochemistry, in liver biopsies is an attractive option for precise localization and quantification of viral proteins with direct access to histological patterns. We describe here a study using a novel immunohistochemical method effective on fixed, archived specimens, including liver biopsies and surgical resection samples. The initial protocol uses a biotin-detection system but can also be used in a polymer-detection system. This protocol offers easy, precise, and strong staining resolution with distinct patterns consistent with the liver pathology, irrespective of the viral HCV genotype examined. This approach provides applications for diagnosis as well as for exploratory pathological studies.

Key words: HCV, immunohistochemistry, envelope protein, chronic infection, hepatocellular carcinoma.

1. Introduction

The HCV genome is a single-stranded RNA molecule of about 9600 bp. It encodes for a large polyprotein that is processed by host and virus proteases into several structural and nonstructural viral proteins (Envelope 1/2, Capsid, p7and NS2, NS3, NS4A/B, and NS5A/B).

The detection of HCV replicative intermediates or virus antigen may be helpful for diagnosis or clinical management of

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patients with HCV infection, and it is of crucial importance for monitoring patients before and after HCV-related liver transplantation. HCV replication level generally seems to be relatively low in infected liver, hampering the detection of HCV particles directly in the liver *(1, 2)*. Detection methods based on HCV RNA amplification, like *in situ* PCR or *in situ* hybridization, do not always detect HCV and can lead to conflicting results concerning localization of viral particles *(3)*, but they remain an interesting tool when used to complement classical methods *(4, 5)*. These methods are probably limited by rapid RNA degradation in tissues and the difficulty of designing efficient probes that overcome the high variability of HCV genomes.

Detection of HCV antigens by immunochemistry in liver biopsies is therefore an interesting option that allows both localization and quantification of viral proteins.

Quite a few antibodies have been raised against hepatitis C antigens and fulfill the conditions for use in immunochemistry (IHC). So far, except for the promising commercial TORDJI 22 and TORDJI 32, no commercial antibody allows specific, reproducible, and efficient staining *(6–8)*, one reason explaining why IHC has failed to become established in routine experiment in diagnostic labs. Among those tested for IHC are antibodies raised against nonstructural proteins (NS3, NS4, and NS5) *(9, 10)* and core or envelope protein *(10, 11)*.

Antibodies raised against envelope protein E2 provided the best efficiency for IHC techniques *(11, 12)*. Even though the region encoding envelope protein encompasses the hypervariable region I (HVR 1) of HCV, the overall conformation of E2 seems to be quite conserved *(13)*. In our experience *(12)*, anti-E2 antibody $D_{4.12.9}$ detected the E2 protein from all genotypes tested, including genotypes 1 to 5, indicating that this antibody is suitable for HCV detection irrespective of its precise genotype. In our personal experience *(12)*, the staining location for anti-E2 immunostaining is mainly cytoplasmic, although occasional perimembrane staining (including cytoplasmic and nuclear membrane) occurs. Staining pattern is mainly coarse granular with a microvesicular pattern (*see* **Fig. 3.1**).

HCV staining patterns appear to differ slightly according to the pathological status of the liver tissue. We observed a very strong staining of hepatocyte membrane, cytoplasm, and perinuclear regions in liver from patients with active HCV-related cirrhosis (Intense plasma and nuclear membrane staining was observed in cases with high inflammatory activity.) In noncirrhotic and nontumoral tissues, anti-E2 staining intensity increased with hepatitis fibrosis state. In HCV-related tumors, staining was exclusively detected within regeneration nodules and confined to hepatocytes whose morphology remained unchanged. Staining appeared in one of two distinct patterns: trabecular throughout the hepatic parenchyma or only in isolated cells.

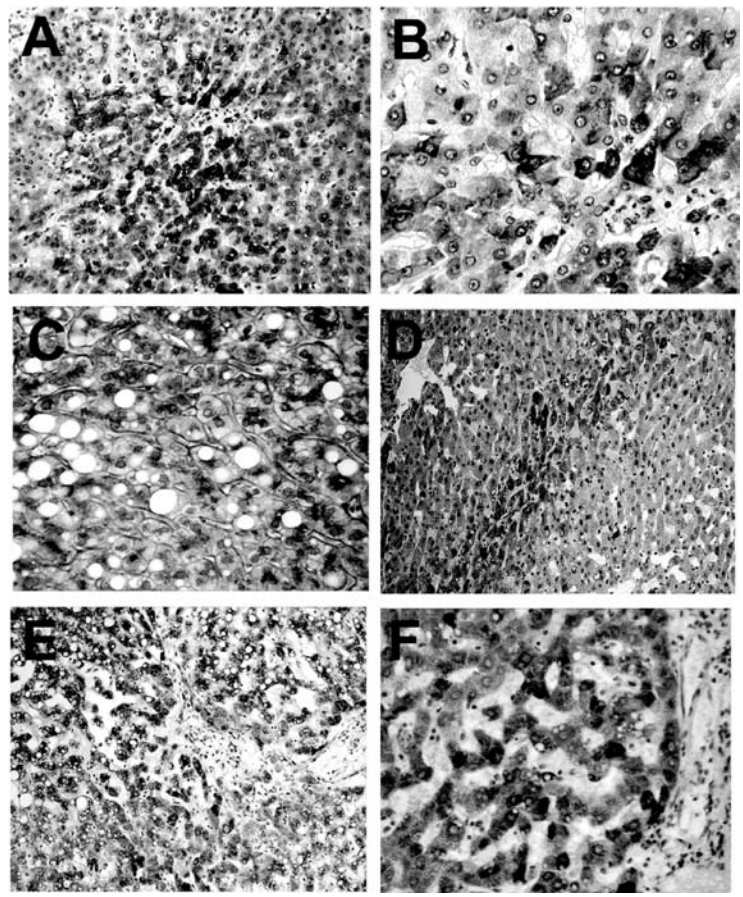

Fig. 3.1. $D_{4.12.9}$ immunostaining of liver sections from HCV patients. **A, B, C.** Severe HCV hepatitis cases. A, B: Patient before hepatectomy, immunostaining with polymer-based assay. C: Immunostaining with classic biotin-based assay. **D, E, F**: HCV-related hepatocellular carcinoma cases. D: Band of hepatocytes with high E2 accumulation among low-intensity stained hepatocytes in nontumorous area, suggesting clonal expansion of infected hepatocytes. E: Cirrhotic tissue surrounding area exhibiting important steatosis and strong E2 expression in cytoplasm and nucleocytoplasmic region. F: Accumulation of E2 in precancerous cirrhotic nodule with more important expression in isolated cells groups.

2. Materials

1. Normal source of samples: Transparietal biopsies or surgical biopsies.
2. Nature of samples: Frozen or formalin-fixed human liver.
3. Xylene dilutions in water.
4. Absolute ethanol dilutions in water: 95%, 70%.
5. Methanol solution (0.3% hydrogen peroxide).
6. Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA).

7. Phosphate-buffered saline (PBS) solution (Sigma).
8. Skimmed milk.
9. Bovine serum albumin (BSA, Sigma).
10. Primary mAb, $D_{4.12.9}$ at 0.2 μg/mL.
11. Secondary antibody, Vectastain® ELITE ABC PEROXYDASE KIT Rabbit IGG (Vector).
12. DAB Substrate kit (Vector).
13. Mayer's hematoxylin.
14. Mounting medium (DAKO Mounting medium, Dako, North America).
15. Slide racks and trays (Fisher Scientific).
16. Microwave oven.
17. Microscope cover glass.
18. Light microscope.

3. Method

1. Deparaffinize tissue (*see* **Note 1**) sections in xylene for 10 min (twice).
2. Rehydrate the tissue in graded ethanol concentrations (100%, 95%, and 70% for 5 min each) and proceed immediately to step 3.
3. Block endogenous peroxidase activities by incubation in methanol solution for 30 min at room temperature (*see* **Note 2**).
4. For unmasking, place the slides in a microwave oven for 15 min in antigen unmasking solution. Set the microwave power high enough to bring the solution to a boil, and then reduce power so that the solution continues boiling for the required time. Allow the slides to cool down for 30 min in the same unmasking solution (*see* **Note 3**).
5. Incubate with PBS solution (5% skimmed milk and 0.1% BSA) for 1 h at room temperature. Do not rinse.
6. Incubate sections overnight with the primary mAb at 4°C. Sections without primary antibody can be used as controls.
7. Wash in PBS three times, 5 min each.
8. Incubate for 30 min with secondary antibody at 5 μl/mL in PBS solution (0.1% BSA).
9. Wash in PBS three times, 5 min each.
10. Amplify signal at 37°C for 45 min (Vectastain peroxydase kit); use PBS solution (0.1% BSA).
11. Wash in PBS three times, 5 min each.
12. Incubate tissue sections with the DAB substrate at room temperature for 5 min,
13. Wash in PBS three times, 5 min each.
14. Counterstain the sections with Mayer's hematoxylin.

15. Dehydrate the section through successive ethanol baths (70% ethanol, 95% ethanol, absolute ethanol, and xylene, 5 min in each solution).
16. Mount slides using standard microscope cover glass and mounting medium.

4. Notes

1. Sensitivity of detection is significantly increased by use of fresh-frozen tissue.
2. Alternatively, a method based on polymer detection can be used with $D_{4.12.9}$ in IHC. It generally offers same sensitivity and a slightly better resolution because it eliminates endogenous biotin interference, the main source of nonspecific background staining. The method differs from the classic protocol in step 3, where we incubate 10 min in DAKO peroxydase block solution (DakoCytomation EnVision + Dual Link System Peroxidase, DAKO, France) and rinse gently with distilled water. For step 8, apply peroxidase-labeled polymer (DakoCytomation EnVision + Dual Link System Peroxidase) to cover specimen. Incubate for 30 min and rinse the slides with the buffer solution provided. Eventually, apply substrate-chromogen solution (DakoCytomation EnVision + Dual Link System Peroxidase) to cover the specimen, add DAB, and incubate for 2–10 min (optimal incubation time can be determined by verifying signal intensity under the microscope). Rinse gently with distilled water from a wash bottle (do not focus flow directly on tissue). Proceed with step 13.
3. During microwave treatment, ensure that solution level is sufficient to cover the tissue section throughout the treatment. Check solution level every 3–5 min.

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