# MORPHOLOGY AND PATHOMORPHOLOGY

# Is Replication of Hepatitis C Virus a Marker of Activity of Infectious Process? (Findings of Polymerase Chain Reaction and Morphological Analysis of Liver Biopsy Specimens)

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Comparative study of replication markers of hepatitis C virus in various biological substrates and evaluation of activity of chronic HCV infection in liver biopsy specimens showed that replication of hepatitis C virus and the number of HCV-infected hepatocytes did not promote liver damage in chronic hepatitis C. These findings indicate that the expression of antiviral reactions by liver parenchyma cells plays the key role in the morphogenesis of HCV infection.

**Key Words:** *chronic HCV infection; virus replication; polymerase chain reaction; liver biopsy; pathomorphology*

The pathogenesis of chronic HCV infection is still poorly studied. Two main mechanisms in the development of infectious process are discussed: direct cytopathogenic effect of the virus and complex immune responses aimed against infected hepatocytes containing foreign HCV proteins [11,14]. To clear this problem, some scientists analyzed the relationships between the amount of virus particles in the serum (viremia) or liver tissue and the type of pathological changes in the liver [8,13].

The fact that viremia reflects virus content in the liver is disputed, because the methods used for evaluation of RNA level in the liver are based on primary extraction from the total hepatocyte RNA, and the results of measurements of the content of the virus in hepatocytes (to be more precise, the number of infected hepatocytes) depend on the sensitivity and specificity of the method. *In situ* hybridization showed [15] that the level of viremia is related to the number of infected hepatocytes in HCV-infected patients with chronic active process (viremia correlated with the percentage of HCV-infected hepatocytes in patients with liver involvement ranging from hepatitis with the minimum activity to cirrhosis). It seems that both variants of chronic HCV infection can co-exhist, and one of the possible approaches is investigation of the correlation between the level of virus "load" and the severity of liver involvement [4,5,12].

We investigated the role of replication of hepatitis C virus in the development of liver damage during chronic HCV infection by pathomorphological and molecular biological methods.

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## MATERIALS AND METHODS

A total of 102 patients with chronic HCV infection (81 men and 21 women aged 15-65 years, mean age 30.66±1.16 years) were observed. Comprehensive studies were carried out in all patients and included biochemical analyses (with measurement of AlAT), serological detection of hepatitis markers by IEA (hepatitis A: anti-HAV IgM; hepatitis B: HBsAg, HBeAg, HBc IgM, HBc IgG, HBsAb, HbeAb; hepatitis C: total anti-HCV and antibodies to Core and NS antigens; and hepatitis D: anti-HDV), and PCR detection of HCV RNA and HBV DNA.

Serum HCV RNA was tested by PCR in 102 cases; in 34 patients complex PCR analysis included HCV RNA testing in 5 biological substrata: blood serum and mononuclears, urine, saliva, and native liver tissue. Qualitative evaluation of viremia was carried out in 19 patients. The measurements were carried out using QIAamp Viral RNA Kit (Cat 29504, QIAGEN), Sibenzim Firm (Novosibirsk), QIAEX II Gel Extraction Kit (Cat 20021, QIAGEN) diagnostic kits, and commercial tests systems from Institute of Epidemiology (Moscow). Viremia was evaluated using test system of Institute of Epidemiology by Boom's method for HCV RNA extraction. The linear range of the test system is from 1000 to 3,000,000 genome copies of viral RNA per ml serum.

Transcutaneous puncture biopsy of the liver was carried out in all 102 HCV-infected patients. Paraffin sections for light microscopy were stained with hematoxylin and eosin in combination with Perls' reaction, by Van Gieson method with Weigert resorcin-fuchsine post-staining of elastic fibers, and periodic acid-Schiff test was carried out. Liver specimens for electron microscopy and immunohistochemical analysis were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2-7.4). Semithin sections were stained with Schiff reagent and Azur II. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a JEM 1010 electron microscope. Activity of hepatitis was evaluated using a 4-point scale: minimum, low, moderate, and pronounced changes [10].

In 34 cases the number of HCV-infected hepatocytes was evaluated by immunodetection of a HCV epitope (immunohistochemical detection of NS3 antigen in paraffin sections). Two-step indirect immunoperoxidase method with streptavidin-biotin visualization system with diaminobenzidine was used. Mouse monoclonal IgG2b antibodies to recombinant NS3 protein (NS3-HCV) (NovoCastra Laboratories Ltd) served as the first antibodies. The data were processed using MS Excel XP statistical analysis and analysis of correlations, regression, and dispersions.

## RESULTS

Genotyping of HCV RNA showed that the majority of patients (60%) were infected with genotype 1b virus, 26% with genotype 2 virus, and 14% with genotype 3a virus. High percentage of intravenous drug addicts among HCV-infected patients many times increased virus passage, which was seen from a rapid shift of the ratio of virus genotypes towards more "favorable" genotypes [1].

Detection of HCV RNA in various biological specimens showed HCV replication in the serum in 77%, in blood mononuclears in 73%, in both serum and mononuclears in 55% cases of HCV monoinfection. In 18% cases virus replication was detected in cells, but not in the serum. HCV RNA was detected in the saliva

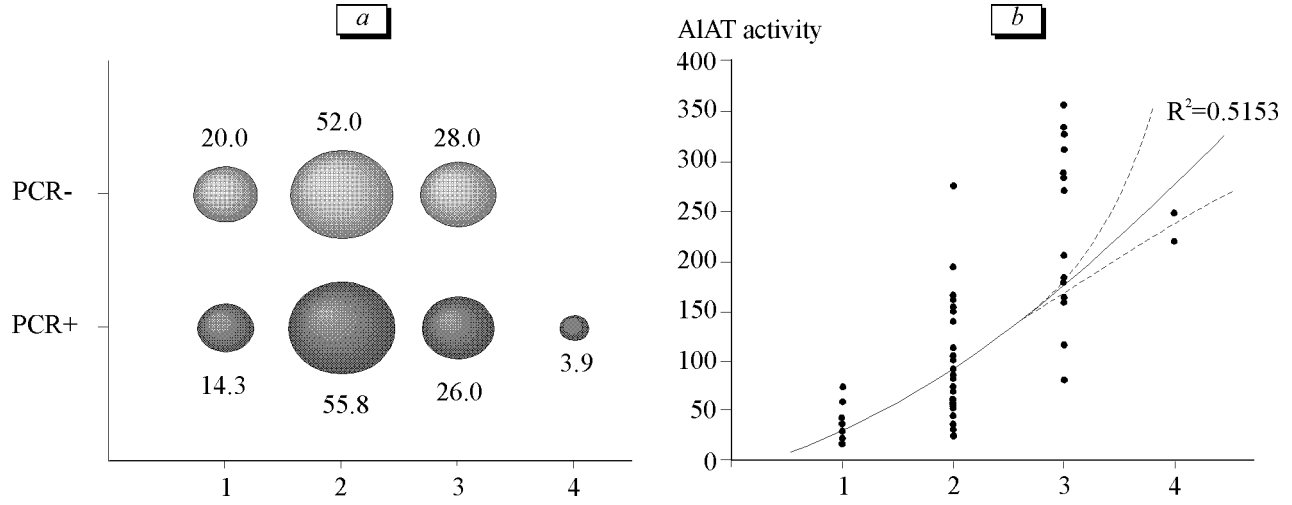


**Fig. 1.** Relationship between activity of HCV infection (1: minimum; 2: low; 3: moderate; and 4: pronounced) in liver biopsy specimens (%) and the presence of hepatitis C virus replication (*a*) and AlAT activity (*b*).

of 7 of 34 patients, in the urine in 5, and in both substrates in 3 patients.

In native liver tissue PCR detected HCV RNA replication in 26 samples (79% cases). Complex studies (blood serum and mononuclears, liver tissue) showed 3 positive results in 52% cases, analysis of 4 substrates (serum and mononuclears, liver tissue, and saliva or urine) was positive in 15% cases, and in 1 case all 5 substrates were positive.

Evaluation of activity of chronic hepatitis C by the analysis of liver biopsy specimens showed minimum activity in 18, weak in 55, moderate in 26, and pronounced activity of the infectious process in only 3 of 102 cases. Minimum activity was characterized by focal degenerative changes in hepatocytes, weak or moderate mononuclear infiltration of the stroma, limited by portal tracts, and the absence of necrosis and necrobiosis foci. In mild hepatitis hepatocyte degeneration was more frequent and often involved several lobules in the biopsy specimen; mononuclear infiltration was usually limited by portal tracts, but segments of lymphodiapedesis formed in the parenchyma; no necrosis and necrobiosis foci were seen.

In disease of moderate activity hepatocyte degeneration was paralleled by monocellular or larger, but solitary, foci of necrosis and necrobiosis or postnecrotic granulomas. Cell infiltration of the stroma was more intense and actively penetrated inside the lobules, destroying the terminal plate and causing hepatocyte disintegration and degeneration (ladder necrosis). High activity of the infectious process was characterized by necrosis (intralobular foci of necrosis and necrobiosis) and intense (sometimes polymorphic) and progressive infiltration disseminating from the portal tracts to the septae and parenchyma.

Analysis of the relationship between serological markers of virus replication (PCR findings) and activity of the infectious process evaluated by examination of liver biopsy specimens showed that the presence of HCV RNA in the serum and/or mononuclear cells was associated with minimum (16%) or low (55%) activity of the process in 71% cases. The absence of HCV RNA was also associated with minimum pathological changes in liver tissue, quantitative parameters virtually coinciding by the degree of activity (Fig. 1, *a*). Statistical analysis showed no correlation between virus replication in the blood and manifestations of structural changes in the liver.

Analysis of HCV replication in the blood (serum and mononuclear cells) and liver tissue by PCR showed positive results in both substrata in the majority (67%) of cases. Replication in the blood alone was detected in 15%, in liver samples alone in 12%, and no markers of replication were detected in 6% cases. Replication of the virus in the blood and liver tissue was associated mainly with minimum or low activity of the pathological process (68% cases). HCV replication in liver tissue in the absence of serological markers of replication was associated with low (minimum or low) activity in all cases. It is noteworthy that higher (moderate) activity was observed in all substrates even without virus replication.

It is noteworthy that 4 of 5 patients with HCV RNA in urine samples had high AlAT activity (116-239) and relatively high (moderate) activity of the infectious process (according to structural changes) in liver tissue. Pronounced structural changes in liver biopsy specimens (moderate activity of the process) were noted in 4 of 7 patients with PCR-positive saliva samples.

Evaluation of viremia in chronic HCV infection showed that the number of HCV genome copies varied from $8.8\times10^4$ to more than 3,000,000 per ml (threshold value for the test system). The majority of the examinees (15 of 19) had high viremia (more than 3,000,000 copies) and minor (from minimum to low activity) structural changes in the liver (10 patients).

Analysis of correlation between infected hepatocytes and degree of liver involvement showed that in the overwhelming majority of cases HCV replication in native liver tissue (80%) and blood samples (83%) (PCR data) was paralleled by immunohistochemical detection of NS3 antigen. Semiquantitative analysis of hepatocytes by expression of NS3Ag showed that liver involvement in chronic hepatitis C did not depend on the number of infected hepatocytes: minimum or low activity of the pathological process in 71% cases was associated with different quantities of hepatocytes expressing NS3 antigen.

An important functional and biochemical markers of hepatitis activity is the level of serum transaminases, primarily AlAT. AlAT activity varied from 10 to 689 U/liter (112.62±10.15 U/liter). In the presence of virus replication in the blood its level was 115.98± 11.42, in the absence of replication 97.54± 19.98 U/liter (differences insignificant). Statistical analysis revealed a positive correlation (R=0.66) between AlAT level and histological activity of chronic HCV infection, but detailed analysis of structural changes in the liver showed that this relationship decreased with increase of the infectious process activity ($R^2$=0.52; Fig. 1, *b*). Low AlAT in chronic hepatitis C can be explained by the fact that AlAT is an intracellular enzyme and increase of its level reflects hepatocyte cytolysis, *i.e.* cell death by colliquation necrosis. Necrosis of this type is extremely rare in chronic hepatitis C; apoptotic transformation or degenerative changes, not paralleled by cytolemma destruction for a long time, are more typical.

Hence, the following basic regularities characterizing the pathogenesis of chronic HCV infection were

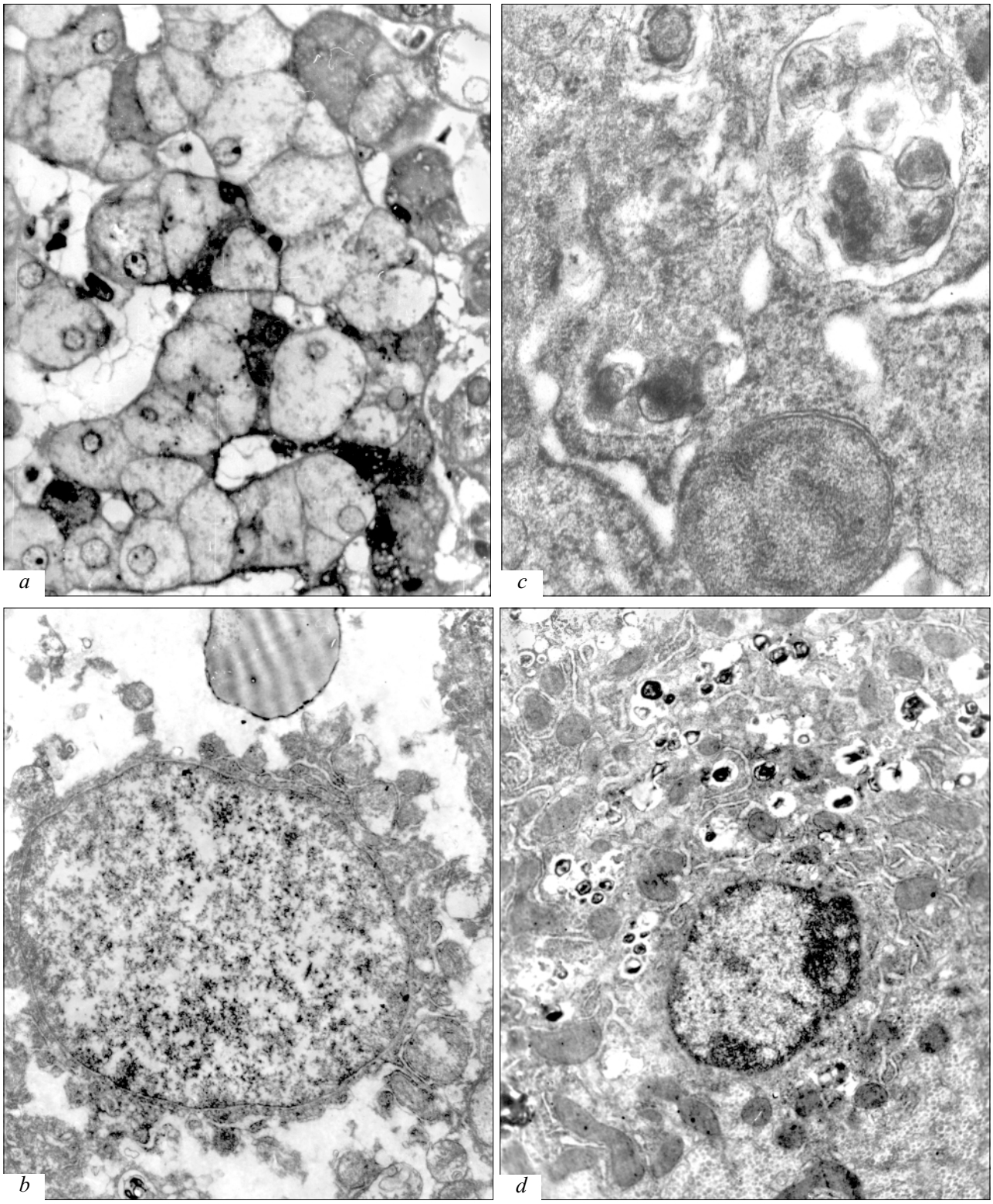


**Fig. 2.** Photooptic and ultrastructural characteristics of parenchymatous liver cells in chronic HCV infection. *a*) empty cytoplasm in the majority of hepatocytes. Semithin section, Schiff reagent and Azur II staining, ×450; *b*) empty cytoplasmic matrix, ×8000; *c*) formation of polymorphic residual bodies as a result of cytopathic reaction, ×25,000; *d*) numerous residual structures in hepatocyte cytoplasm, ×4000.

detected: no correlation between the presence of HCV RNA in the blood and type of structural changes in the liver; no relationship between the presence of HCV RNA simultaneously in the blood and liver tissue and the level of viremia, on the one hand, and activity of the infections process, on the other. Liver involvement

does not depend on the number of infected hepatocytes (according to immunodetection of HCV NS3Ag).

On the whole, liver involvement in chronic HCV infection is not directly related to the number of infected hepatocytes or the presence of HCV RNA in the blood and liver tissue. The morphogenesis of chronic hepatitis C can explain this paradox. When examining liver biopsy specimens, we distinguished a new form of degeneration, cell involution, predominating in chronic hepatitis C [3]. The main marker of this term of degeneration is devastation of the cytoplasmic matrix with intact nuclear compartment and perinuclear foci of intracellular regeneration (Fig. 2, *a*, *b*), indicating that this state is reversible. Complex morphological examinations of serial sections and paired biopsy specimens showed that devastation of the cytoplasm indicates the period of hepatocyte sanation after cytopathogenic action of the virus (Fig. 2, *c*, *d*). Hepatocyte sanation is realized by exocytosis of residual structures without lysis of HCV-infected cells [2] in the presence of intact structure and function of the cell and organ, in contrast to immunomediated cytodestruction often associated with hepatocyte cytolysis.

Since the immune system is unable to control hepatitis C RNA-virus characterized by great genetic variability and numerous variants [6,9], the defense reactions of the hepatic parenchymatous cells and their mechanisms of antiviral defense formed in the course of evolution are expressed. Infected hepatocytes can actively participate in antivirus response, and this fact is an important amendment to the dogmatic theory, regarding infected hepatocytes as merely "victims" of infection [7].

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