p53 immunocytochemistry and *TP53* gene mutations in patients with chronic hepatitis C virus (HCV) infection

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Abstract: Chronic infection with hepatitis C virus (HCV) is regarded as a risk factor for hepatocellular carcinoma (HCC), mostly in patients with liver cirrhosis. Present study aimed at evaluation of cellular expression of p53 protein, genetic *TP53* changes in liver samples and anti-p53 in serum of patients with chronic hepatitis C virus infection. The expression of p53 protein were analysed by immunocytochemistry in liver biopsies from adult patients with chronic, long-lasting hepatitis C. In order to detect *TP53* mutations, PCR/SSCP and sequencing were performed. Antibodies against p53 in serum were determined using enzyme immunoassay (ELISA). In two out of 14 examined patients *TP53* point mutations were detected in the liver samples. In the first patient, a substitution of C to T was demonstrated in position 1 of the codon 250, resulting in substitution of proline by serine. The other patient carried a substitution of C to G in position 13274 of the intron 6. The patient carrying mutation in the codon 250 demonstrated morphological traits of liver cirrhosis and had high number of p53-immunoreactive cell nuclei in tissue. None of the patients manifested elevated titres of serum anti-p53. In the liver, significant positive correlations were disclosed between expression of p53 on one hand and *grading* and *staging* on the other. A negative correlation was disclosed between cellular expression of p53 and duration time of infection. In conclusions, genetic changes in *TP53* can be detected also in non-neoplastic lesions linked to chronic HCV infection.

Key words: chronic hepatitis C, histopathology, p53 immunocytochemistry, *TP53* gene expression

Introduction

Chronic infection with hepatitis C virus (HCV) is regarded as a risk factor for hepatocellular carcinoma (HCC), mostly in patients with liver cirrhosis [1]. There are also evidences for the association of HCV, mostly genotype 1b, with HCC without the intermediate step of cirrhosis [2-4]. The liver cell lines constitutively expressing full-length or truncated versions of the HCV genome show a high incidence of chromosome instability [5]. The potential candidates for the group of oncogenes in development of HCC include at least three HCV proteins, *i.a.*, the core (C protein, capsid protein), NS3 and NS5A [6]. *p53*

gene (*TP53*) is considered the prototype tumor suppressor gene and is the most extensively studied tumour suppressor implicated in HCC [7]. Testing of the gene status represents worldwide one of method of diagnosing and establishing prognosis in HCC [4,7-11]. Wild type *TP53* codes for a nuclear phosphoprotein, which controls proliferation of normal cells, DNA repair and apoptosis [12,13]. The protein coded by *TP53* manifests a short half-life and is expressed in very small amounts, precluding the chance to detect it using immunocytochemical techniques [14]. Control of *TP53* takes place also by the alternative splicing while an additional protein product (p47) controls ubiquitination, degradation, and cell localization of p53 [15]. In neoplastic cells augmented concentration of p53 protein is detected, absent from normal cells [12]. Mutant p53 proteins are easily detectable by immunocytochemical methods due to their abnormally extended half-life [16]. The mutated

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p53 protein of an abnormal function may be responsible for selective expansion of hepatocytes in the process of cancerogenesis [17]. Frequency of *TP53* mutations in human HCC varies in geographic zones between 10% and 60% of the cases [18-22]. Mutations of *TP53* are reported most frequently in countries with high risk of HCC development, including China and South Africa, in which they are linked to exposure to aflatoxin B1 (AFB1) and viral infections (mainly with hepatitis B virus, HBV) [8,23-25]. In the countries most mutations are at one nucleotide pair (Arg→Ser) of codon 249 [23,26,27]. A dramatic multiplicative effect on HCC risk was demonstrated for a combined exposure to HBV and 249[SER] p53 mutations [28]. In countries of a low exposure to AFB1 (Japan, European countries), the most frequently described defects of *TP53* in HCC involve point mutations [19,29,30]. Loss of heterozygosity (LOH) [29] and individual gene deletions were also reported [30]. A common polymorphism at codon 72 resulting in the change of arginine to proline has recently been correlated to HCC [22]. Most of investigators are of the opinion that genetic alterations in *TP53* are related to rather late stages of hepatocellular carcinogenesis, which are common for various etiologies of chronic hepatitis [29,31]. Few reports only indicate that overexpression of p53 and mutations of *TP53* may manifest themselves also in non-neoplastic hepatic lesions [26,32-34], and upon exposure to AFB1 presence of 249[SER] *TP53* mutation is also detected [35]. Still no ideal molecular marker of various HCC developmental stages is available for epidemiological and diagnostic studies even if suitability is stressed of plasma tests for the presence of *TP53* mutations, including quantitative studies (qSOMA) [9,11].

In view of the scanty number of studies on the role of p53 protein in carcinogenesis with the *in vivo* background of HCV infection, present study aimed at evaluation of *TP53* status (genetic changes in liver samples, cellular expression of p53 protein, anti-p53 in serum) in patients with long-lasting (approximately 20 years) chronic HCV infection. It was planned to find out whether the long-lasting hepatitis C *per se* may be responsible for the increased incidence of *TP53* gene mutations in genomic DNA of patients with a high potential risk of HCC development (long-term HCV infection, liver cirrhosis following HCV infection, 1b genotype of HCV) but manifesting no clinical and histological traits of neoplastic transformation in the liver.

Material and methods

Liver samples. Studies were performed on archival biopsies of liver, obtained from adult patients (n=16) with documented chronic, long-lasting hepatitis C and with active replication of the virus, confirmed serologically (all the patients were anti-HCV and HCV RNA positive). The duration time of the infection was estimated basing exlusively on the infection factor HCV (blood transfusion, extensive operation, long hospitalization). The 16 biopsies originated from patients of the Department of Infectious

Diseases, Poznan University of Medical Sciences, in whom the biopsies were performed in 2005-2006. All the patients were seronegative for both HBs and HBe antigens, and for IgM antibody against human cytomegalovirus (HCMV), against Epstein-Barr virus (EBV) and against HIV-1 and HIV-2 by standard enzyme immunoassays (ELISA). Other causes of liver damage (*e.g.* α_1-antitrypsin deficiency, Wilson's disease, alcohol dependency) were ruled out. All the patients had exhibited elevated serum alanine transaminase level (normal level below 40 IU/l) for at least 6 months. All the patients were seropositive for antibody to HCV by ELISA (HCV version 3.0 AXYM System, Abbott). Quantitation of HCV RNA was conducted using AMPLICOR HCV™ test, version 2.0 (ROCHE, Mannheim, Germany) with sensitivity of 600 IU/ml. The test was standardized against the WHO International Standard for HCV RNA. Before liver biopsy none of the patients was subjected to anti-viral therapy. Written informed consent was obtained from each patient before liver biopsy, and approval for the study was granted by the institution's Ethical Committee.

The control group I samples were obtained from normal livers of serologically HCV-, HBV-, HCMV- and EBV-negative organ donors and normal livers from tissue microarray panel (Cybrdi Inc.; Maryland, USA) (n=17; mean age: 58±2 years). These normal controls were without morphological evidence of pathology and were negative controls for immunocytochemical detection of p53 protein. The control group II consisted of the archival paraffin embedded fragments of livers with hepatocellular carcinoma (HCC, n=18; mean age: 52±4 years), among which human HCC tissue microarrays documented cases of different HCC, PCNA confirmed types (Cybrdi Inc.; Maryland, USA). Only one patient from group II was both HBV- and HCV-positive, in the remaining patients their serological status related to HCV and HBV infections remained unknown. Control group II provided positive control for immunocytochemical detection of p53 protein. Control for the studies on serum anti-p53 in patients with chronic HCV infection included sera of 10 healthy adult blood donors (mean age: 32±2 years).

Liver biopsy specimens from the patients with chronic hepatitis C and control group I were fixed in 10% buffered formalin, embedded in paraffin for purposes of light microscopy. Histopathological lesions were evaluated, following the classical H+E staining as well as silver technique and tri-chromate technique, according to Masson and periodic acid-Schiff with diastase pretreatment. At least 10 sections were prepared from each biopsy or from control liver fragments. Each tissue specimen was evaluated basing on a numerical scoring system for the grade of portal/periportal necroinflammation (G1=0-4), for the grade of lobular necroinflammation (G2=0-4) and for the stage of fibrosis (S=0-4), as proposed by Scheuer [36]. Histological evaluation was performed independently by two experienced histopathologists (WB and JB).

Isolation of DNA from liver. DNA for genetic studies was isolated from liver biopsies obtained from 14 patients with chronic hepatitis C in the course of diagnostic biopsy of the organ. The biological material was immersed in 2 ml sterile H_2O and stored in a Venosafe tube, containing K_2EDTA (final concentration of 0.1%). Samples for further studies were frozen at the temperature of -20°C. DNA was isolated using QIAamp DNA Blood Mini Kit (QIAGEN, Germany), as recommended by the manufacturer. Amount and purity of the isolated DNA were estimated by agarose gel electrophoresis in 0.8% agarose gel supplemented with ethidium bromide (0.5 µg/ml). The control involved DNA isolated from blood cells of healthy donors (n=10; mean age: 32±2 years).

Amplification of DNA fragments using polymerase chain reaction (PCR). PCR reaction was performed in a Biometra thermocycler in the final volume of 10 µl. The reactive mix contained approximately 50 ng DNA, 1 µM primers, 200 µM dNTP, 2 U Taq polymerase (DYNAzyme II DNA polymerase, 1mM spermidine in the attached polymerase buffer, containing: 10mM Tris-

HCl pH 8.8; 1.5mM MgCl$_2$; 50mM KCl; 0.1% Triton X-100) (FERMENTAS, Canada). The samples were subjected to preliminary denaturation for 1 min at the temperature of 94°C and, then, subjected to 35 cycles of amplification. Every cycle consisted of three stages, including denaturation of double stranded DNA at the temperature of 94°C for 30 s, primer annealing to single stranded DNA for 30 s at the temperature of 58°C and synthesis of complementary DNA at the temperature of 72°C for 1 min. In the terminal, 35th cycle the stage of complementary DNA synthesis at the temperature of 72°C was extended to one minute. The amplification products were analysed following their electrophoretic separation in 2% agarose gel, supplemented with ethidium bromide (0.5 μg/ml), at 4 V/cm and their visualization under UV light.

Single stranded conformation polymorphism (SSCP) analysis and direct DNA sequencing for *TP53* mutations. PCR-SSCP analysis was carried out for exons 5-9 from approximately 1μg genomic DNA of the *TP53* gene, in which most frequently changes are observed in HCC [18], using the following primers (5'→3'):
Exon 5: CTC TGT CTC CTT CCT CTT CC and CAG CCC TGT CGT CTC TCC
Exon 6: GCT GCT CAG ATA GCG ATG G and CCC TTA ACC CCT CCT CCC
Exon 7: TGC TTG CCA CAG GTC TCC and CAG ACG AGG CCA GTG TGC
Exon 8: GAC CTG ATT TCC TTA CTG CC and GAA TCT GAG GCA TAA CTG C
Exon 9: CAG GGA GCA CTA AGC GAG and TCC ACT TGA TAA GAG GTC CC
The primers were designed using Primer Selekt, DNAstar software. They were produced by the Institute of Biochemistry and Biophysics (IBB) PAN, Warszawa, Poland. PCR product in the volume of 10 μl was mixed with 12.5 μl formamide (chemical denaturation) and 2.5 μl staining buffer for SSCP (final concentrations of components: TBE-0.25x, 2% Ficol-400; 0.01% bromophenol blue and 0.01% xylene cyanole FF). Subsequently, the DNA-containing mixture was subjected to thermal denaturation at the temperature of 95°C for 10 min and cooled in ice. The next stage included electrophoretic separation in 10% polyacrylamide gel (PAA) in 0.5% TBE buffer, at 180V for approximately 12 hours. The separated DNA bands were stained with silver salts. For this purpose the DNA-containing PAA gel was incubated with slight shaking, passing it consecutively through 10% ehanol solutions for 15 min and 1% HNO$_3$ for 10 min. Then, it was stained with 0.2% AgNO$_3$ solution and 0.1% formaldehyde for 30 min and developed using 3% Na$_2$CO$_3$ and 0.05% formaldehyde solution. The reaction was blocked using 10% acetic acid for 15 min. At the next stage PAA gel was incubated in 4% glycerol for 20 min and dried at the temperature of 75°C. Between the consecutive solutions, the gel was rinsed with high amounts of MiliQ water.

Direct PCR sequencing. DNA fragments which demonstrated distinct migration from that in control samples were amplified by PCR using the same primers. Products of PCR reaction were purified using QIAquick PCR Purification KIT (QIAGEN, Germany) as suggested by the manufacturer and were subjected to automated sequencing. The studies were performed in the Faculty Laboratory of Molecular Biology Techniques, Adam Mickiewicz University in Poznań. Sequencing analysis took advantage of the 3130x Genetic Analyzer (Applied Biosystem) using Big Dye Terminator v 31 cycle sequencing kit. Results of sequencing were analyzed using Chromas software. Comparison of nucleotide sequences and translation of nucleotide sequences into amino acid sequences were performed using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST).

Concentration of serum autoantibodies reactive with p53 (anti-p53) in patients' blood. Anti-p53 were estimated in serum of 16 patients using p53 ELISA^{PLUS} (Autoantibody Kit, Calbiochem^R). The control included 10 sera obtained from healthy donors. The assay was interpreted by determining the relative p53-autoimmune index, which was calculated as follows:
ABS$_{450}$(sample) – ABS$_{450}$ (cut-off)/ABS$_{450}$(calibrator) – ABS$_{450}$(cut-off) = p53 Autoimmune Index
Cut-off for the obtained curve at the absorbance level of 0.15 (ABS$_{450}$) amounted to 0.125 U/μl. When a dilution was taken into account, the cut-off value in tested sera of patients with hepatitis C and of control subjects amounted to 12.5 U/μl.

p53 protein immunocytochemistry. For immunocytochemistry, 5 μm thick sections were cut and mounted onto SuperFrost/Plus microscope slides. Mouse anti-human monoclonal antibodies were employed, directed against p53 protein (ready-to-use) (clone DO-7; DAKO, Glostrup, Denmark), which recognized the epitop located within amino acids 35-45 of the N-terminus of wild and mutant types of the p53 protein. After deparaffination and rehydration of preparations in xylene and a row of alcohols of a decreasing concentration (2x100%, 90%, 85%, 80%, 70%), they were washed in tap water (10 min), activity of endogenous peroxidase was blocked using 1% H$_2$O$_2$ (30 min). Subsequent washing in a distilled water was followed by incubation of the preparations in a normal goat serum (1:20, DAKO) (30 min). Then, the sections were treated with primary MAb at night at 4°C and for 1 h at room temperature. After washing in phosphate-buffered saline (PBS) 3×3 min, the sections were incubated with the secondary biotinylated link anti-mouse and anti-rabbit IgG (DAKO) (20 min) and with the streptavidin-biotin-peroxidase complex (LSAB2, DAKO) (20 min). They were sequentially washed in PBS 3×3 min, incubated (7 min) with the HRP substrate, 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) (DAKO) in 0.05 M Tris-HCl buffer, pH 7.6, supplemented with 0.001% H$_2$O$_2$, washed in tap water and in distilled water (2x10 min), dehydrated in a row of alcohols of an increasing concentration (70%, 85%, 90%, 95%, 2x100%), and equilibrated with xylene. The preparations were sealed under coverslips in the Canada balsam. Selected preparations were counterstained with hematoxylin (2 min). The studies followed the classical ABC technique [37] with microwave-oven pretreatment for antigen retrieval. Positive reaction manifested, in at least three sequential sections, as a dark brown or black precipitate in the cell nucleus and/or cytoplasm. Control reactions were based on substituting specific antibodies with normal sera of the respective species in 0.05 M Tris-HCl, pH 7.6, supplemented with 0.1% BSA and 15 mM sodium azide (negative control).

Semiquantitative evaluation of the results and statistics. The content of p53 protein in liver biopsies were calculated by the semiquantitative technique and reported as follows: 0=0% positive cells; 1= individual positive cells (<10%); 2=10-25% positive cells; 3≥26-50% positive cells; 4≥51-75% positive cells, according to [38], evaluating 10 microscopic fields at magnification of 400x. The final result represented mean score for the 10 fields. Expression of p53 protein was evaluated only in hepatocytes (cell nuclei and/or cytoplasm). All the preparations were examined under an OLYMPUS B-2 light microscope. The descriptive statistics included calculation of mean, median values, standard deviation and standard error of the mean (SEM) for quantitative traits. In order to determine statistical significance of variations in the expression intensity of the p53 protein, we first calculated the mean values of staining scores for liver biopsy groups. In comparison of quantitative traits the Mann-Whitney's U test for was used for non-parametric independent data. Correlations between data rows were determined employing Spearman's rank correlation index. Probability values less than 0.05 were considered significant.

Results

At the time of biopsy the mean age of patients (10 men and 6 women) was 41±4 years (range: 19 to 65 years). At the time of liver biopsy, the mean duration

Table 1. p53 protein immunocytochemical expression, *TP53* mutation and anti-p53 detected in each patient in relation to the clinical and histopathological features of HCV-infected patients

Case no.	Age (years)	Sex	HCV genotype	Duration of HCV infection (years)	*Grading*[1] G1	G2	*Staging*[1]	SSCP (exon, intron)	Base exchange (codon)	Anti-p53 (U/µl)	p53 protein[2]
1.	29	F	1b	9	1	2	1	-	-	7.0	0
2.	34	M	3a	unknown	1	1	1	-	-	6.1	1
3.	25	F	1b	25	1	1	1	-	-	6.9	0
4.	57	F	1b	12	3	3	4	-	-	5.7	1
5.	27	M	1a	27	0	1	1	-	-	7.1	0
6.	41	M	3a	26	2	1	0	-	-	7.0	0
7.	28	M	1b	unknown	1	1	1	-	-	6.5	0
8.	55	M	1b	19	1	3	2	-	-	9.4	1
9.	20	M	1b	19	0	1	1	-	-	5.4	1
10.	53	M	1b	unknown	3	2	4	7	C→T; pos. 1 (250)	6.0	4
11.	25	F	3a	10	2	2	2	intron 6	C→G; pos. 13274	7.6	1*
12.	59	F	1b	25	1	2	1	-	-	7.8	0
13.	19	F	1b	19	1	0	1	-	-	7.4	0
14.	54	M	1b	30	0	1	3	-	-	9.6	0
15.	60	M	1b	14	3	3	4	nt		10.8	1
16.	65	M	4c/4d	14	2	2	4	nt		7.1	2*

G1 – portal/periportal inflammatory activity (*grading*); G2-*grading* in lobules; S-advancement in fibrosis (*staging*); [1]score according to [36]; [2]Score: 0=0% cells; 1=individual positive cells (<10%); 2=10-25% positive cells; 3=26-50% positive cells; 4=51-75% positive cells according to [38]; * – nuclear+cytoplasmic expression in hepatocytes; nt – not tested; pos. – position

of HCV infection was 19±2 years. The majority (69%) of the patients included in our study were infected by HCV genotype 1b (Table 1).

p53 DNA analysis

Mutational analysis by PCR-SSCP showed two from 14 examined HCV-infected liver samples with aberrant migration pattern indicative of genomic aberration and only in exon 7. Examples of results obtained in DNA single strand conformational polymorphism DNA (SSCP) related to exon 7 of *TP53* are shown in Fig. 1. Differences in migration of single strand DNA fragments are marked with arrows. The separation in 10% polyacrylamide gel was repeated as shown in the two panels. Direct PCR sequencing analysis was performed in the samples with aberrant SSCP bands to characterize the genetic aberration. In the patient No. 10 a C to T substitution was demonstrated in the first position of codon 250 (CCC→TCC), resulting in interchange of proline to serine. In the patient No. 11 a substitution of C to G was demonstrated in position 13274 of the intron 6 (Table 1).

Anti-p53

All blood samples in HCV-infected patients tested for the presence of anti-p53 demonstrated values below the threshold level (Table 1). In the curve of the test at the absorbance of 0.15 the cut-off value amounted to 0.125 U/l. Taking into account the dilution, the final cut-off value was 12.5 U/µl. Mean value of p53 antibodies in HCV-infected patients was 7.34±0.37 U/µl and in control group was 6.80±0.42. No significant difference of studied serum p53 antibodies was estimated between the HCV-infected patients and control patients (p=0.28) (data not shown).

p53 immunocytochemistry

The total immunocytochemical detectability of p53 protein amounted to 8/16 (50%) of tested liver biopsies in patients with chronic hepatitis C. There were 6 samples which showed positive nuclear p53 expression but in less than 10% of the cells, one sample which showed 10-25% p53-positive cells and one sample which showed
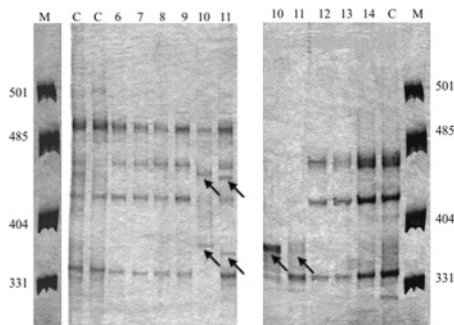
Fig 1. Examples of results obtained in PCR-SSCP analysis of *TP53* gene exon 7 in control (C) samples and HCV-infected liver samples (patients No 6-14). Lane M shows electrophoretic pattern of size marker (pUC). The arrows indicate changes in migration of individual pre-denatured PCR products in 10% polyacrylamide gel (they indicate the genetic *TP53* changes in liver samples No 10 and 11). The separation in polyacrylamide gel was repeated. Sample identification corresponds to numbers of patients in Table 1.

>50% p53-positive cells (Fig. 2A). In two cases cytoplasmic plus nuclear localization of p53 was observed in chronically HCV-infected livers (cases no 11 and 16) (Table 1). In the control group I no p53 protein could be detected in any of examined samples (Fig. 2B). In the material of liver with HCC (control group II) presence of individual or very numerous p53-immunoreactive cell nuclei was disclosed in 11/18 patients (61%) (Fig. 2C and Fig. 2D). In two cases of HCC cytoplasmic reaction for the protein prevailed (Fig. 2D).

Correlations between expression of p53 protein in HCV infected patients and selected clinical data

Positive Spearman's correlations could be disclosed between tissue expression of p53 protein and intensity of inflammatory lesions (*grading*) both in portal spaces (G1) (r=0.548; p=0.03), and in hepatic lobuli (G2) (r=0.551; p=0.03) as well as between intensity of p53 protein expression and *staging* (r=0.705; p=0.002). A negative Spearman's correlation was disclosed between tissue expression of p53 protein and duration of the infection (r=-0.570; p=0.04) in patients with chronic hepatitis C. No correlation could be detected between expression of p53 and age of the patients (r=0.368; p=0.16).

Characteristics of patients with chronic hepatitis C and TP53 aberrations

In the patient No. 10 (infected by HCV genotype 1b), with C→T transition in position 1 of the codon 250 (exon 7) (CCC→TCC; interchange of proline to serine), the most pronounced nuclear expression of p53 protein was noted (above 50% of cells) among the 16 examined patients by immunohistochemistry. In histopathological examination the patient manifested traits of liver cirrho-

sis and a significant *grading* in portal spaces. In the patient, duration of HCV infection could not be determined. Despite the documented point mutation, no elevated levels of anti-p53 antibodies could be detected in serum of the patient. The second patient, No. 11 (infected by HCV genotype 3a) in molecular tests demonstrated C→G transversion in position 13274 of the intron 6 of *TP53*, and in the immunocytochemical examination positive nuclear and cytoplasmic location of p53 was demonstrated but only in few (below 10%) hepatocytes. In the patient a moderate *grading* (2 points) and *staging* (2 points) was disclosed and duration of the infection reached 10 years. No elevated titers of anti-p53 antibodies were demonstrated.

Discussion

Multiple reports have appeared worldwide which described genetic alterations in *TP53* also in codons other than codon 249 of the gene (*e.g.*, codons 136, 156, 157, 213) and most of them involved missense point mutations [8,18,19,29,30]. Mutations of *TP53* with interchange of a single amino acid were identified in over 100 different codons [33]. Aberrations were also observed which affected introns of *TP53* [30]. In HCC described by European authors mutation in the codon 249 was detected in a single case [19]. Few studies have suggested that losses and gains of chromosome regions may differ in HCCs caused by HBV as compared to HCV infection [39]. Few studies have dealt with incidence of genetic aberrations in *TP53* in patients with non-neoplastic liver pathology or with risk factors prompting development of HCC (*e.g.* chronic viral infections) or the reports relate to selected exons of *TP53* [32,34,35,40]. No mutations in the codon 249 were detected in North American patients with chronic hepatitis and/or liver cirrhosis as compared to 5/6 positive samples of non-neoplastic liver from Mozambican patients (HBV infection and AFB1 exposure) [40]. Studies on *TP53* alterations in normal liver samples demonstrated a linear relationship between incidence of mutations in the codon 249 and the extent of exposure to AFB1 [35]. Akyol *et al.* in 21 different pathological entities of non-tumoral liver, demonstrated overexpression of p53 protein in 35% specimens, significantly more frequent in patients with steatohepatitis [32]. Studies in non-tumorous tissues of patients with Wilson's disease and hemochromatosis documented higher frequency of genetic aberrations (transversions and transitions) in both codons 249 and 250 of *TP53* in the patients, as compared to healthy controls [34]. Examination of genetic lesions in present study pertained the exons 5-9 of *TP53*, in which gene aberrations were described most frequently [18,19,23,26,27,38]. Genetic alterations and p53 protein expression were examined at the tissue level and
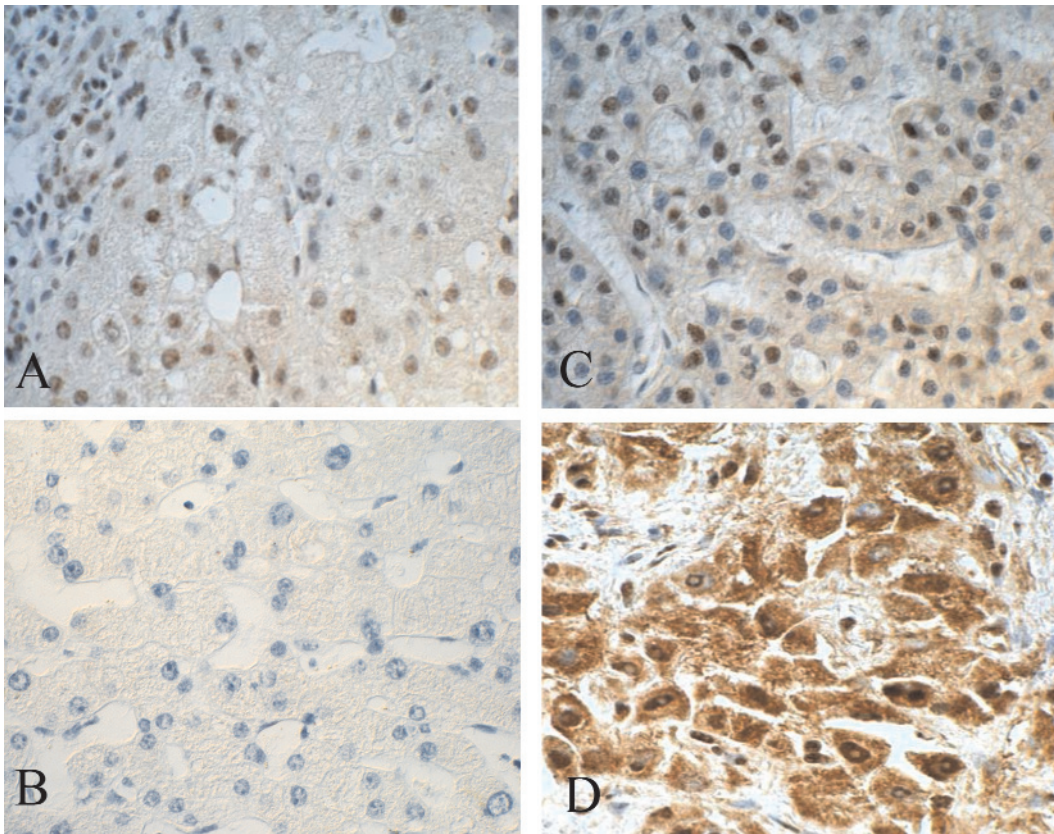
Fig. 2. The slide shows fragment of chronic HCV-infected patient's liver and *TP53* mutation (Patient No. 10) with immunocytochemical localization of p53 protein in hepatocyte nuclei (A), normal liver (control group I) with no positive staining of p53 protein in hepatocytes (B), immunocytochemical localization of p53 protein in many cell nuclei in HCC patient (C); and HCC patient with p53-positive cell's cytoplasm and nuclei (D). (Strept)avidin-biotin peroxidase complex (ABC) method. Hematoxylin counterstain (original magnification x400).

presence of anti-p53 antibodies was looked for in sera of the patients. In the present study alterations in *TP53* nucleotide sequence were documented in two patients (in the codon 250 and intron 6). In the first patients a transition of C→T was detected in position 1 of the codon 250 (exon 7) (CCC→TCC) which caused substitution of proline by serine and presence of p53 protein in cell nuclei of numerous hepatocytes. The patient manifested traits of liver cirrhosis and was infected with HCV genotype type 1b. The other patient in molecular studies demonstrated transversion C→G in position 13274 of the intron 6 in *TP53* gene while immunocytochemical examination detected p53 localization in cell nuclei and cytoplasm of few hepatocytes. C:G to T:C transversions and C:G to T:A transitions were demonstrated also in the codon 250 in a patient with Wilson's disease [34]. It seemed significant that the mutations were detected at the stage which preceded a potential neoplastic process, allowing to undertake appropriate therapeutic procedures. Correlations were described between specific *TP53* mutations and less favourable prognosis or response to therapy [33]. A significantly abbreviated survival characterized patients with HCC and with *TP53* mutations [8]. Studies on clinical significance of detec-

tion of so variable alterations in *TP53* have indicated that a heterogenous cellular response to carcinogenic factors (including viral infections) is related to both inherent ability of individual *TP53* gene mutants to inhibit function of wild type p53 and to the status of the remaining allele of *TP53* [33]. However, an enhanced risk for developing HCC exists when the active HCV infection (present in all patients examined in this study) is accompanied by mutations in *TP53* [28].

Examination of p53 protein alone in other non-neoplastic patients with HCV infection (but still free of HCC) using an immunoluminometric technique have demonstrated overexpression of p53 in almost 18% of the patients [4]. In this study, application of ABC technique has confirmed both nuclear and cytoplasmic localization of p53 protein. However, in most of the patients the expression has been low or has been absent. In the examined material, the definitely highest proportion (above 50%) of cells with positive immunocytochemical reaction in cell nuclei of hepatocytes has been demonstrated in the patient with mutation in the codon 250 of *TP53*. This is consistent with the general observation that mutations of *TP53* often stabilize the protein, leading to higher steady-state p53 levels than in wild

type cells and that this can be detected by immunocyto-chemical techniques [16]. It is also assumed that protein of nuclear localization has lost its transcriptional activity [17]. However, it has been shown that not all mutations stabilize p53 and, thus, application of exclusively immunocytochemical techniques in studies of *TP53* expression may underestimate incidence of mutations in tumors with deletions, frameshift or nonsense *TP53* mutations [17,41]. On the other hand, in present study mutations in *TP53* have been detected not always in the cases with expression of the protein in cell nuclei. Presence of p53-immunopositive cells in the absence of sequence mutations was observed also by other authors [24,30]. Overexpression of p53 protein, usually associated with point mutations in *TP53* gene, was detected in almost half of HCC cases studied in Europe [20]. Other European studies on non-neoplastic diseases of the liver (liver cirrhosis due to HBV and HCV infections), conducted by Koskinas *et al.*, have failed to demonstrate expression of the protein in the tissue [31].

In this study we have demonstrated also a cytoplasmic localization of p53 protein in individual cells both in the patient with alteration in the intron 6 of *TP53* and in the patient with no detectable genetic alterations in exons 5-9 of *TP53* but with morphological traits of liver cirrhosis. In two our cases of HCC cytoplasmic reaction for the protein (control group II) was observed. Multiple studies were devoted to cellular localization of both the wild and the mutated p53 protein [15,42-44]. Various types of human tumors, including HCC, have demonstrated abnormal p53 cytoplasmic localization, and this is associated with tumour metastasis and poor prognosis [43]. p53 protein localized in the cytoplasm is thought to be less responsive to signals that would normally induce its nuclear retention [42]. Cytoplasmic sequestration of p53 in tumor cells is linked to a poor long-term patient survival and may be substantial fraction of tumors that do not have mutated p53 [45]. In none of the patients examined in this study have elevated serum titers of anti-p53 been detected despite presence of *TP53* mutations and/or immunocytochemical detection of p53 protein in livers of the patients. This is consistent with observations of Readle *et al.* on a large group of patients with HCV-related HCC and non-neoplastic lesions [46]. The authors and several other investigators state that presence of anti-p53 is highly specific for the already developed tumor and that incidence of the antibodies varies between 7 and 20% HCC [46-48]. Other studies on prognostic significance of anti-p53 antibodies in diagnosis of malignant tumors, including HCC, have shown that just in HCC and in breast cancer elevated serum anti-p53 signify less advantageous prognosis as to survival of the patients (analysis of Kaplan-Meier). Diagnostic sensitivity of the marker increases when it is applied in line with conventional tumor markers [49].

It is difficult to interpret the negative correlation between expression of p53 protein in our patients and duration of the infection. The positive correlations between expression of mutated p53 and of inflammatory lesions (*grading*) and advancement of liver fibrosis (*staging*) in our HCV-infected patients prove that histological lesions provide a much more reliable marker of the disease progression than duration of the infection (not always reliably documented). The positive relationships between p53 expression and *grading*, both in cases of non-neoplastic liver damage [32] and in already developed HCC have been described by other authors [20,31]. However, other studies on HCC have failed to document correlations between p53 expression and age or *grading* [21]. As indicated by long-term studies on patients with mutations of *TP53*, who did not develop HCC in the course of 5 years, biological significance of genetic alterations in *TP53*, detected in non-neoplastic patients remains probably distinct than that in patients with HCC [26], but it seems important to cover the patients with point mutations in *TP53* and with overexpression of tissue p53 with a particular care and to treat them as a group carrying the risk of development of HCC and/or of other tumors.

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