p21/Wafl/Cipl cellular expression in chronic long-lasting hepatitis C: correlation with HCV proteins (C, NS3, NS5A), other cell-cycle related proteins and selected clinical data

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Abstract: Studies indicate that proteins of hepatitis C virus (HCV) disturb expression of cell-cycle-related proteins. A disturbed cell-cycle control is a hepatocellular carcinoma (HCC) risk factor in patients with HCV-related liver damage. The present study aimed to analyse the cellular expression of p21/Wafl/Cipl (p21) in long-lasting chronic hepatitis C (CH-C), its correlation with the key oncogenic HCV proteins (C, NS3, NS5A), other cell-cycle-related proteins (PCNA, Ki-67, cyclin D1, p53) and selected clinical data. Archival liver biopsies, obtained from patients with CH-C, normal livers, and hepatocellular carcinoma (HCC) specimens were analysed by immunocytochemistry and ImmunoMax technique. In CH-C overexpression of p21 protein was demonstrated. Positive correlations of p21 protein expression in CH-C involved age of the patients, *grading*, and liver steatosis. Moreover, expression of p21 correlated significantly with expression of p53 protein, of D1 cyclin and Ki-67. Although Ki-67 antigen was related to p21 expression, only Ki-67 expression proved to be directly related to liver *staging*. Expression of the NS3 protein, which prevailed in CH-C patients, manifested correlation with p21 expression, and that of cyclin D1. In presence of preserved potential for regeneration, overexpression of p21 indicates inhibition of cell cycle in hepatocytes, which probably plays a protective role for the chronically damaged cells. Out of the three HCV proteins only NS3 seems to affect control of p21 protein expression in *in vivo* infection. Nevertheless, the studies indicate that neither expression of p21 protein nor that of viral NS3 protein can serve as a marker of progression of CH-C to HCC *in vivo*.

Key words: Long-lasting chronic hepatitis C, p21/Wafl/Cipl and other cell cycle-related proteins, NS3, NS5A and C protein, ABC and ImmunoMax methods

Introduction

Hepatitis C virus (HCV) is considered to represent the principal causative agent of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). The search continues for reliable (serum and tissue) markers of progression manifested by HCV-related chronic hepatitis to hepatocellular carcinoma [1,2]. A disturbed cell-cycle control is a HCC risk factor in patients with HCV-related liver damage [3]. Results of studies indicate alterations in expression of cell-cycle-linked proteins with the block in hepatocyte cell cycle in G1 phase [4,5]. An experimental proof is available for the role of three HCV proteins: the core protein, nonstructural protein 3 (NS3), and NS5A in control of the cell cycle [6]. The most important host proteins which control cell cycle are thought to include the wild-type p53 and p21 proteins [7]. The p21/Wafl/Cipl (p21) protein is a downstream target effector of wild-type p53 [8]. The best recognised role of p21 involves inhibition of cyclin complexes (CKI) with numerous cyclin-dependent kinases: CDK4/D, CDK6/D and CDK2/E, which control introduction of the cell to the cycle and start of DNA replication [9]. p21 co-localizes and

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binds also with the proliferating cell nuclear antigen (PCNA) [10] and it may directly inhibit PCNA-dependent DNA replication [11]. In response to mitogens, p21 is induced during G1 phase and, thus, appears to perform a role in normal cell cycle progression in the absence of growth inhibitors [12]. p21 protein was also shown to block cell cycle in G2 phase, which is dependent on retinoblastoma protein [13]. In tissue culture systems, p21 is up-regulated in proliferating cells [12], whereas, in other cell types, it is induced during senescence and terminal differentiation, and it is thought to play a key role in down-regulating CDK activity in these settings [14]. The p53-dependent expression of p21 plays a central role in cell growth regulation and apoptosis [8]. p21 can be induced also in a p53-independent manner [12,13]. In normal tissue expression of p21 to high levels is not dependent on p53 and confirms that induction of p21 by DNA-damaging agents does require p53 [12].

In cases of chronic hepatitis C (CH-C) more p21-immunopositive cells were demonstrated in cirrhotic lesions of the liver as compared to regions with inflammatory lesions [2]. The other studies in HCV infection point to correlation between p21 expression and fibrosis stage, *grading* and serum HCV RNA levels and they document block in cell cycle in G1 phase [4]. In HCC a decreased expression of p21 was detected mainly as result of a mutation of p53 [15,16]. Recent studies indicate also that proliferation of human HCC cells requires JNK1-dependent p21 downregulation [17]. Evaluation of p21 expression is also of importance in distinguishing patients with a risk of a more rapid progression from chronic hepatitis to HCC [1].

The *in vitro* studies on interactions between HCV proteins and *p53* and/or *p21* genes provide variable results. The most numerous interactions were documented for the HCV core protein (capsid, C protein) [18-21]. The originally incompatible results of studies on stimulation of p21 expression by the core protein [20,21] or on inhibition of the expression [22], seemed to be explained by the subsequent papers. The effects of interactions with p53 and/or p21 were found to be dependent both upon the concentration of the core protein and on its localization in the cell [19,23]: low concentration of the core protein stimulated activity of p53 while its high concentration decreased the activity [19]. The cytoplasmic form of core protein augmented p21 expression (via activation of p53) and the mature form in the cell nucleus inhibited it (the p53-independent pathway) [23]. In an another study inhibition of p21 synthesis was demonstrated by the core protein situated in the cytoplasm [24]. Out of the non-structural HCV proteins, NS3 can promote growth [25] but NS5A protein can either promote [26] or inhibit growth via up-regula-

tion of p21 [27]. Few *in vivo* studies are available which attempted to correlate cellular expression of p21 and p53 protein expression in CH-C with expression of the potentially oncogenic HCV proteins [28]. Moreover, the literature data are inconsistent as to the tissue expression of cell cycle-related proteins and the relationships between clinical data and tissue expression of HCV proteins *in vivo*. Examining effects of active HCV replication on alterations in cell cycle proteins of the host cells *in vivo*, we attempted to find out if cellular expression of p21/Wafl/Cipl protein correlates with expression of potentially oncogenic HCV proteins (C, NS3, NS5A) and with clinical data in long-lasting chronic hepatitis C. We undertook an attempt to examine the relationship between expression of HCV proteins and expression of the typical proliferation-associated antigens, i.e., Ki-67 and PCNA (present in all phases of cell division), cyclin D1 (engaged in control in phases G1-S of the cell cycle) and p53 protein, which controls proliferation of normal cells, DNA repair and apoptosis. We aimed also to assess the potential relationship between cellular expression of the cell-cycle proteins and selected clinical data in long lasting CH-C (mean duration of HCV infection approximately 20 years).

Material and methods

Liver samples. Studies were performed on 29 archival biopsies of liver, obtained from adult patients (16 men and 13 women) 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 biopsies originated from patients of the Department of Infectious Diseases Poznan University of Medical Sciences, in whom the biopsies were performed in 2005-2008. All the patients were seronegative for both HBsAg and HBeAg, and for IgM antibody against cytomegalovirus (CMV), against Epstein-Barr virus (EBV) and against HIV-1 and HIV-2 by standard enzyme immunoassays (ELISA). Other cases 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. The duration time of the infection was estimated basing exclusively on the infection factor HCV (blood transfusion, extensive operation, long hospitalization). 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 negative control samples were obtained from livers of serologically HCV-, HBV-, HCMV- and EBV-negative organ donors and normal livers from tissue microarray panel (Cybrdi Inc.; Maryland, USA) (n=13). These normal controls were without morphological evidence of pathology. Appropriate positive control for cell cycle-related protein expression involved the livers with HCC from patients and tissue microarray panel (Cybrdi Inc.)

Table 1. Epidemiological, histological and clinical data of the patients with chronic long-lasting hepatitis C.

Chronic HCV patients (n=29)					
Demographics		Liver histology*		Clinical data	
Male:Female	16:13	cirrhosis (S4)	34%	Viral genotype 1b	76%
Age (y)	42.1±3.1	G1	1.7±0.2	ALT (U/l)	83.0±10.8
Duration time of infection (y)	20.9±2.1	G2	1.9±0.2	AST (U/l)	72.0±9.2
		G1+G2	3.6±0.3	AFP (IU/ml)	6.4±2.4
		S	2.2±0.3	HCV RNA (IU/ml)	769740.0±304227.7
		Steatosis	1.3±0.1		
		AFP	1.9±0.3		

Legend: Values shown mean value and standard error of the mean (SEM); y – years; * – histopathological scoring (see section: Materials and methods); G1 – grade of portal/periportal necroinflammatory; G2 – grade of lobular necroinflammation; S – stage of fibrosis; AFP – alphafetoprotein

(n=18). Only one patient from HCC group was HCV-positive, in the remaining patients their serological status related to HCV infection remained unknown.

Liver biopsy specimens fixed in 10% buffered formalin, embedded in paraffin were evaluated histologically by two experienced histopathologists (WB and JB), following the 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 livers. 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) [29].

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 (1) the p21$^{Wafl/Cipl}$ protein (clone SX118; DAKO, Glostrup, Denmark; in 1:50 dilution), (2) the human Ki-67 antigen (clone MIB-1) (DAKO, 1:2), (3) the PCNA antigen (DAKO, 1:200), (4) cyclin-D1 (clone: DCS-6; DAKO; ready-to-use dilution), (5) the p53 protein (clone DO-7; DAKO; ready-to-use), (6) capsid C protein (CHEMICON International, Inc., 1:50), (7) NS3 protein and (8) NS5A protein (both from NOVOCASTRA Labs., 1:50). The studies followed the ABC technique and/or the ImmunoMax technique (in case of p21, NS3, NS5A, C proteins) as described earlier [30,31]. In ImmunoMax technique, the key reaction involved 8 min incubation with biotinylated tyramine (PerkinElmer Life Sciences, Inc., 1:50) at room temperature. 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 (internal negative control).

Semiquantitative evaluation of the results. The frequencies of staining and the contents of HCV proteins, p21, Ki-67 and PCNA antigens, cyclin D1, and p53 protein in liver biopsies obtained using ABC and/or ImmunoMax technique were independently measures by two investigators. The interobserver and intraobserver variation was <10%. Expression of HCV proteins and cell cycle-related proteins (cell nuclei and/or cytoplasm of hepatocytes) was calculated taking into account mean proportion of immunopositive cells in 10 different areas of each specimen. Positive nuclear or cytoplasmic staining was evaluated by counting a total of at least 1000 cells. Steatosis of liver was also semiquantitatively appraised, scoring 0 when no steatosis was noted under a light microscope, and annotating grades 1 or 2 when, respectively, <30% of hepatocytes or 30-70% of hepatocytes were affected [32]. Alphafetoprotein (AFP) expression in hepatocytes were calculated 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; 5=76-100% positive cells) [33], evaluating 10 microscopic fields at magnification of 400×. The final result represented mean score for the 10 fields. All the preparations were examined under an OLYMPUS B-2 light microscope.

Statistical analysis. 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 of the proteins, we first calculated the mean values of staining scores for liver biopsy groups. In comparison of quantitative traits the Mann-Whitney's U test was used for non-parametric independent data. Correlations between data rows were determined employing Spearman's rank correlation index and Kruskal-Wallis test. Probability values less than 0.05 were considered significant.

Results

At the time of biopsy the mean age of patients (16 men and 13 women) was 42±3 years (range: 19 to 74 years) and the mean duration of HCV infection was 20.9±2.1 years. The majority (76%) of the patients included in our study were infected by HCV genotype 1b. Epidemiological, biochemical and virological data of patients are summed up in Table 1.

Liver grading, staging and steatosis in HCV-infected patients.

Mean values of *grading, staging*, steatosis of liver and AFP expression in tissue are listed in Table 1. A statistically significant positive Spearman's correlation was documented between grade of portal/periportal necroinflammatory (G1) (r=0.369; p=0.048) (data not shown) and total *grading* (G1+G2) on one hand and steatosis on the other (r=0.362; p=0.05), between total *grading* on one hand and *staging* on the other (r=0.725; p=0.001) (Fig. 1).

Expression of HCV proteins (C, NS3 and NS5A)

Cytoplasmic expression of all the three HCV proteins prevailed. Nuclear localization of C, NS3 and NS5A

Table 2. Expression of HCV proteins and of p21/Wafl/Cipl (p21) protein and the remaining cell-cycle linked proteins in patients with chronic hepatitis C (CH-C), negative control (CONTR.) and positive control (hepatocellular carcinoma, HCC). The numbers indicate mean percentages and standard error of the mean (SEM).

	CH-C	CONTR.	HCC
C protein	20.7±4.2	0.00	0.6±0.6
NS3 protein	71.1±4.4[a]	0.00	1.7±1.7
NS5A protein	33.0±5.7	0.00	1.7±1.7
p21 protein	23.6±4.1[b]	0.00	1.2±0.6*
	p=0.001		NS
p53 protein	5.6±2.4	0.00	16.8±5.6[NS]
		NS	p=0.004
PCNA	12.6±2.4	0.5±0.2	22.1±1.9**
	p=0.001		p=0.001
Ki-67 antigen	10.2±1.8	4.1±1.7	21.2±3.5***
	p=0.003		p=0.001
cyclin D1	4.4±1.1[c,d]	2.0±0.9	10.6±3.4[NS]
	NS		NS

Legend: a – p=0.001 NS3 *vs.* C and NS5A in CH-C; b – p=0.0001 p21 *vs.* p53; c – p=0.004 cyclin D1 *vs.* PCNA; d – p=0.002 cyclin D1 *vs.* Ki-67; * – p=0.0001 p21 in CH-C *vs.* HCC; ** – p=0.002 PCNA in CH-C *vs.* HCC; *** – p=0.004 Ki-67 antigen in CH-C vs HCC; NS – statistically non significant between the respective groups

was observed only in individual cells (Fig. 2). A statistically significant most pronounced expression in hepatocytes pertained the NS3 protein, both as compared to C protein and NS5A. No quantitative differences were disclosed in expression of C protein and NS5A protein (Table 2). A positive Spearman's correlation was documented between expression of NS3 protein and NS5A protein in livers of HCV-positive patients (r=0.428; p=0.03) (data not shown). In the negative control no HCV protein expression was detected. In HCC expression of HCV proteins was noted in only one patient (Table 2 and Fig. 2).

Expression of cell cycle-related proteins

Expression of p21/Wafl/Cipl. Expression of p21 protein was detected in 22/25 (88%) of the examined liver samples; the immunocytochemical reaction used to be markedly intense and present exclusively in cell nuclei (Fig. 3). On the average, the expression was observed in 23.6±4.1% cells (Table 2). It was significantly higher than expression of the protein in both controls (p=0.0001) and higher than expression of p53 protein (p=0.0001). A positive, statistically significant Spearman's correlation was disclosed between expression of p21 and that of p53 (r=0.397; p=0.049), between expression of p21 and Ki-67 (r=0.437, p=0.03), and also between expression of p21 and that of cyclin D1
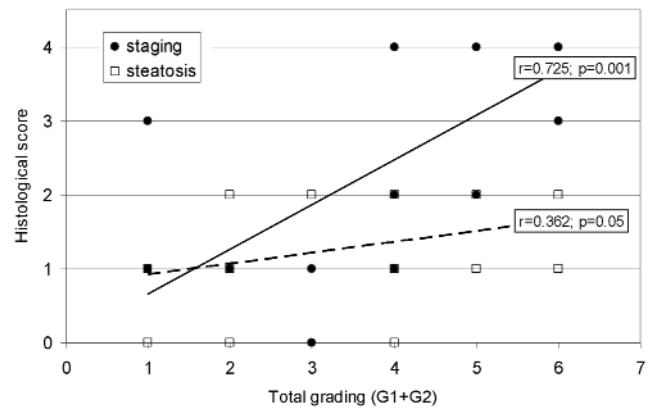

Fig. 1. Spearman's correlation between the total liver *grading* (G1+G2) and for the stage of liver fibrosis (*staging*) and liver steatosis.

(r=0.532; p=0.007) (Fig. 4). Moreover, the expression of p21 protein demonstrated correlation both with total *grading* (G1+G2), and with liver steatosis (r=0.413; p=0.04; r=0.452; p=0.02, respectively) (Fig. 5). In the negative control no p21 expression was noted. In HCC both nuclear and cytoplasmic positive reaction for p21 was observed rather in individual cells and only in 4/18 examined tumours (Fig. 3). The average percentage of the expression amounted to 1.2±0.6 cells (Table 2). It was significantly lower than the expression noted in hepatitis C (p=0.001). No significant differences were seen between expression of p21 in the negative control and in HCC (p=0.298).

Expression of p53 protein. As a rule p53 protein was detected in cell nuclei although individual cells were detected with the cytoplasmic reaction (Fig. 3). The average expression of p53 involved less than 10% cells (Table 2). A positive Spearman's correlation was observed between expression of p53 and p21 in HCV-infected patients (r=0.397; p=0.049) (Fig. 4). Expression of p53 failed to correlate with *grading*, *staging*, and/or steatosis. In the negative control no expression of p53 protein was noted. In HCC numerous p53-positive cells were observed in a few patients (Fig. 3). In HCC the average expression of p53 amounted to 16.8±5.6% cells and even if the result was higher than that in CH-C patients, the difference proved to be insignificant (p=0.06). On the other hand, a significantly higher p53 expression was detected in HCC group, as compared to the negative control (p=0.004) (Table 2).

Expression of Ki-67, PCNA and cyclin D1. Expression of Ki-67, PCNA and cyclin D1 was seen exclusively in cell nuclei. The frequency of PCNA manifestation in liver biopsies amounted to 92% patients and on the average expression of the antigen could be seen in 12.6±2.4% cells. Ki-67 was demonstrated in all liver biopsies in the mean proportion of 10.2±1.8%
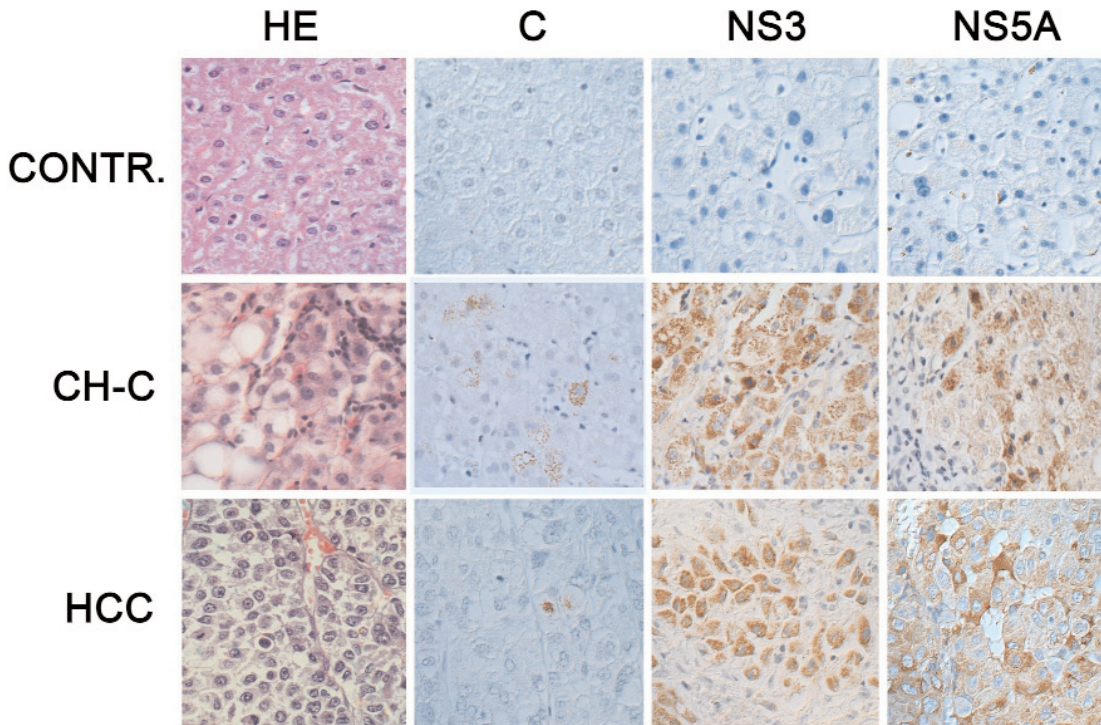
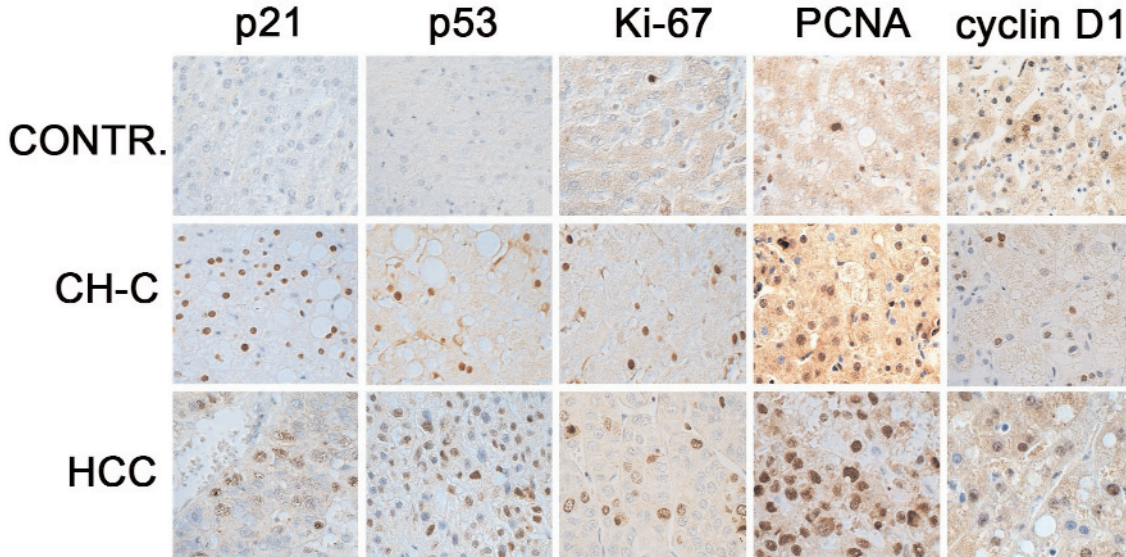**Fig. 2.** Micrographs of normal livers (CONTR.), chronic hepatitis C (CH-C), and hepatocellular carcinoma (HCC). First panel (left) shows hematoxylin/eosin (HE)-stained sections to demonstrate normal histology (CONTR.), characteristic enlarged hepatocytes with swollen, partially vacuolised cytoplasm, often with the evident fatty degeneration in chronic hepatitis C (CH-C) and acinar/pseudoglandular pattern and nuclear crowding in hepatocellular carcinoma (HCC). The next panels show immunocytochemical staining for HCV proteins: C (core), NS3 and NS5A. Original magn. ×400.

Fig. 3. Micrographs of normal livers (CONTR.), chronic hepatitis C (CH-C), and hepatocellular carcinoma (HCC). Panels show immunocytochemical staining for cell-cycle related proteins: p21WAF-1/CIP-1, p53, PCNA, Ki-67 antigen and cyclin D1. Original magn. ×400.

cells. In the group of patients no significant differences could be detected in cellular expressions of PCNA and Ki-67. However, as compared to the negative control, the expression was higher in CH-C patients (Table 2). Expression of cyclin D1 was noted, on the average, in 4.4±1.1% of studied cell nuclei in 11/24 (46%) liver biopsies (Table 2). Expression of cyclin D1 was significantly lower than expression of PCNA (p=0.004) or Ki-67 antigen (p=0.002). In the case of Ki-67 the more intense immunocytochemical reaction was observed as compared to those detecting PCNA and cyclin D1 (Fig. 3). No significant reciprocal correla-
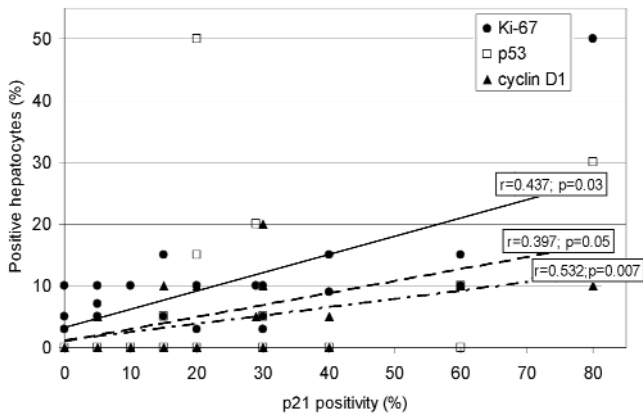
Fig. 4. Spearman's correlation between expression of p21 protein and of Ki-67 antigen, p53 protein and cyclin D1 cellular expression.

Fig. 5. Spearman's correlation between expression of p21 and of the total *grading* (G1+G2) and for the liver steatosis.
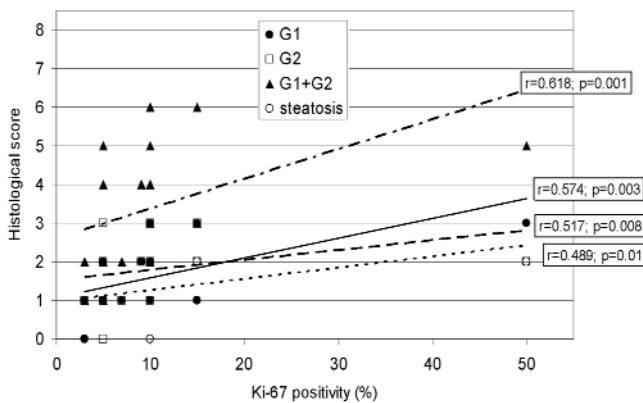

Fig. 6. Spearman's correlation between expression of Ki-67 antigen and of portal/periportal necroinflammation (G1), the grade of lobular necroinflammation (G2), total *grading* (G1+G2) and for the liver steatosis.

tions could be documented between expressions of proliferation-associated antigens. Significant positive correlations were detected between expression of the Ki-67 antigen and *grading*, both in portal spaces (G1) (r=574; p=0.002), and in the lobules (G2) (r=0.517; p=0.008), and total *grading* (G1+G2) (r=0.618; p=0.001). Expression of Ki-67 proved to be directly related to steatosis of the liver (r=0.489; p=0.01) (Fig. 6) and to the *staging* (test of Kruskal-Wallis, p=0.046) (Fig. 7). In the negative control only individual Ki-67-positive or PCNA-positive cells were noted, as compared to numerous Ki-67-positive and PCNA-positive in HCC (Fig. 3). A significantly higher expression of both proliferation markers was noted in HCC, as compared to CH-C and control (Table 2). In the case of cyclin D1 individual cells with expression of the protein were observed in the negative control material while in HCC expression of the protein was observed in relatively numerous cell nuclei (on the average in 10.6±3.4% of the cells) (Fig. 3). In HCC expression of the protein was higher than in patients with CH-C and

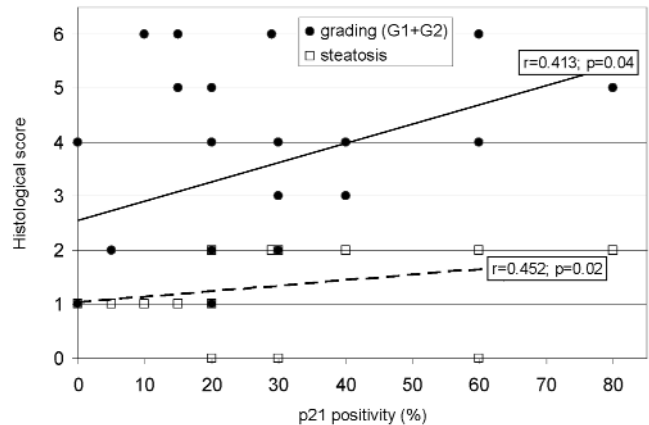than in the negative control but the difference was statistically insignificant (Table 2).

Correlations between HCV proteins and proteins of the cell cycle

Expression of the NS3 protein manifested significant correlation with expression intensity of both p21 (r=0.399; p=0.048), and that of cyclin D1 (r=0.455; p=0.02) (Fig. 8). No correlation could be noted between expressions of HCV proteins and Ki-67 antigen, PCNA and p53.

Correlations between studied cell cycle markers, HCV proteins and clinical data

None of the studied HCV proteins manifested correlation with patient's age, duration of HCV infection, serum levels of HCV RNA and AFP or tissue AFP expression. Out of cell cycle proteins only expression of p21 manifested a direct relationship to patient's age (r=0.449; p=0.02). The remaining cell cycle proteins demonstrated no significant correlations with the clinical data.

Discussion

Most frequently, the oncogenic properties of HCV proteins are linked to their potential for translocation to cell nucleus and to the control of cell cycle through their cooperation with cell-cycle related proteins and to control of apoptosis. Several studies point to the relationship between subcellular localization of HCV proteins, their levels, specific molecular form, presence of specific domains and their effect on oncogenesis-linked mechanisms [6]. The search continues for reliable (serum and tissue) markers of progression manifested by HCV-related chronic hepatitis to hepatocellular carcinoma [1,2].
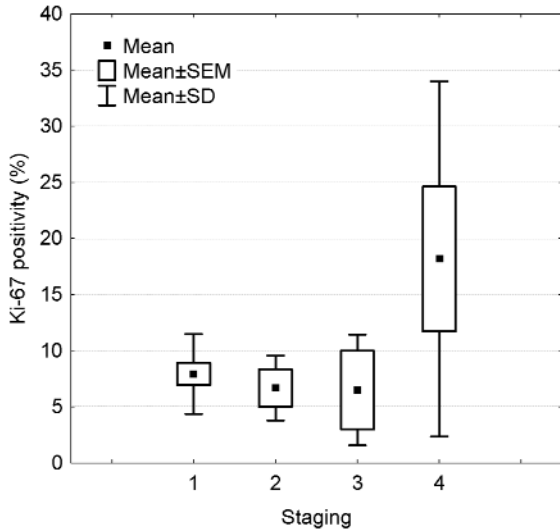
Fig. 7. Comparison of Ki-67 antigen expression with respect to liver fibrosis score (*staging*) (test of Kruskal-Wallis).

Fig. 8. Spearman's correlation between expression of NS3 protein and p21 and cyclin D1 cellular expression.

Present studies have demonstrated mostly cytoplasmic expression of all three viral proteins (C, NS3 and NS5A) in patients with CH-C, which is consistent with the earlier observations [32] and with the currently accepted knowledge on HCV replication [3]. Expression of NS3 protein has prevailed. In the currently examined group of patients similarly to the earlier studies [32] no correlation could have been detected between expression of HCV proteins and histological lesions or selected clinical variables. This remains in agreement with results of other authors [28]. On the other hand, we have noted that expression of NS3 protein was directly related to expressions of p21 and cyclin D1. We have not been able to confirm the *in vitro* results demonstrating positive [20,21] or negative relationships [22,24] between core protein and p21 protein. We also couldn't demonstrate any correlation between NS5A protein and p21 protein expression [26,27]. Similarly to our results, a strong positive correlation between NS3 and p21 in non-neoplastic HCV-positive lesions adhering to HCC has been demonstrated by few authors [28]. In general, NS3 is known to be linked to the neoplastic transformation of normal hepatocytes in chronically infected patients [6]. In *in vitro* experiments it was demonstrated that NS3 specifically inhibited activity of *p21/WAF1* promoter, especially when combined with HCV core protein [25]. In turn, studies of other authors point to an inhibitory effect of various HCV proteins (*e.g.* core protein), but not NS3, NS5A or NS5B on expression of p21 at the translation level [34]. Thus, the effect of NS3 protein on cell cycle-related proteins (including p21) in HCV-infected cells does not always involve inhibition and the effect probably depends upon reciprocal co-operation of all HCV proteins. In present study we have corroborated the
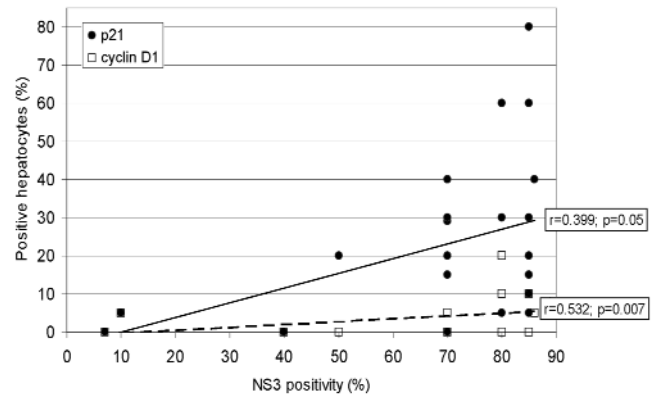
positive correlation between expression of p53 and p21, similarly to the results of some authors obtained in HCV infection [28]. In our HCV-positive material p21 has been the most frequently manifested cell cycle protein (24%). Our observations indicate that if expression of p21 was so frequent, the hepatocytes contained functional wild-type p53 protein. We can confirm the earlier data that the p53-dependent mechanism of p21 expression is observed more frequently as a result of DNA damage, *e.g.*, due to infection with HCV, than due to the p53-independent mechanism in "normal cells" [12]. Nevertheless, the augmented expression of p21 together with increased production of nitric oxide synthase (NOS-2) without parallel detection of p53 was detected in chronic hepatitis C [35]. In HCV infection subsequent authors demonstrated also elevated expression of p21 and p53 proteins, but without reciprocal correlations [5]. Studies of still other authors proved that expression of the protein may represent an independent prognostic index of HCC development in humans [2]. In our patients with CH-C, expression of p21 has been directly related to expression of Ki-67 antigen, similar to other authors [36]. Similarly to the latter authors we have not been able to demonstrate expression of p21 in "normal" livers [36]. In liver biopsies with a particularly intense expression of p21 and Ki-67, up-regulation of p21 may reflect simply increased numbers of proliferating cells, which is observed during progression of hepatic diseases and in liver regeneration [1,5,12,36]. Positive correlation between the p21/MIB-1 coefficient and number of dysplastic lesions was demonstrated in patients with highly advanced chronic hepatitis of various etiology (including HCV infection) [1]. Other studies indicate that positive correlations between p21 expression and end-stage liver disease scoring point to inhibition of hepatocyte proliferation in the course of evolution of chronic hepatitis to liver cirrhosis and that this is accompanied by up-regulation of p21 [37]. Even if

other authors also observed a correlation between p21 and *staging* in CH-C, in the same study they detected positive correlation between the proliferation marker, Mcm-2 and *staging* [5]. In the material studied by us expression of p21 has manifested correlation with *grading* and with steatosis of liver but not with *staging*. Similarly to other authors we have noted positive correlation between *staging* and expression of Ki-67 [5]. This indicates at least a partially preserved potential of hepatocytes for regeneration in long-lasting CH-C. On the other hand, however, chronic hepatitis and increased proliferation of hepatocytes may promote development of HCC. The examined by us patients have demonstrated a low expression of Ki-67 (10%), and histopathological evaluation of cirrhosis has pertained 10/29 (34%) patients. In the HCC group expression of p21 has been low as a rule, accompanied by variable expression of p53 positivity in livers of patients with HCC, which points to the lowered production of p21 in this type of human tumours. The result corroborates conclusions of other authors [16,28]. However, except of a single case, we cannot specify etiology of HCC in our material. Other studies on patients with CH-C documented positive relationship between *staging* and accumulation of replicative senescent cells, evaluated by estimation of activity shown by senescence-associated beta galactosidase [38]. In our studies expression of p21 correlated also with age of the patients, which seems to confirm the observations that senescent cells in liver *in vivo* are associated with older age [38]. As mentioned above, we have not been able to demonstrate relationships between expression of p21 and *staging* [2,37], probably due to the relatively low number of liver cirrhosis cases (approximately 30%) in the studied material. However, it should be noted that in our patients already total *grading* alone has correlated with *staging*. Similarly to expression of p21, expression of Ki-67 also has correlated with *grading* and liver steatosis. This suggests that HCV infection at this stage on one hand induces augmented proliferation of hepatocytes and, on the other, increases expression of p21, which is supposed to protect the cell by inhibition of cell cycle and preventing against neoplastic transformation.

On the basis of the positive correlation between expressions of p21 and cyclin D1 in our material we can suggest that p21 represented rather a stimulator of cyclin/CDK complex formation than it inhibited activity of the complexes. It has even been demonstrated that expression of cyclin D1 induced transcription of p21, which did not block the cell cycle but rather stabilised the cyclin D/cdk4 complex [39]. The positive correlation between p21 and cyclin D1 is consistent with reports showing that p21 assists in securing nuclear localization and acts as an assembly factor for cyclin D/cdk complexes [40]. Expression of cyclin D1 is observed in several tumours in humans, but contradictions are noted as to its prognostic importance [41,42]. In HCC overexpression of cyclin D1 was associated with a well-differentiated histology and a low Ki-67 labeling index and may be an early event in hepatocarcinogenesis [41]. Other studies showed that overexpression of the *cyclin D1* gene resulted in the rapid growth of a subset of HCC [42]. Our results in patients with CH-C have documented a very low expression of cyclin D1 (below 5%), a higher one in patients with HCC (10%) but the differences have not been significant.

Our studies on expression of p53 have confirmed mostly nuclear localization of the protein. We assume that the positive expression of p53 provides an evidence for presence of a protein form with a higher half-life, which may result from mutation of *p53* [43], or from action of agents damaging DNA of host cells with no detectable mutations of the protein [44]. Our earlier observations confirmed presence of point mutations in *p53* in 2/14 examined livers of CH-C patients [31].

In our material we have observed no correlations between expression of cell-cycle proteins on one hand and duration of HCV infection, level of HCV RNA, serum and/or tissue concentration of AFP on the other.

In summary, our present studies have demonstrated mostly cytoplasmic expression of HCV proteins. Expression of NS3 protein has prevailed in CH-C patients. In the group of patients overexpression of p21 protein was detected, as compared to normal liver and HCC. Positive correlations were disclosed between expression of p21 in CH-C on one hand and patient's age, *grading*, and steatosis on the other. Moreover, expression of p21 was significantly correlated with expressions of p53, cyclin D1 and Ki-67. A direct relationship was shown for expression of p21 and that of NS3 proteins.

Our results indicate that, upon partially preserved potential for liver regeneration, elevated expression of p53-dependent expression of p21 provides evidence for inhibition of cell cycle in numerous hepatocytes, which probably has a protective significance for chronically injured or older hepatocytes. Out of the examined HCV proteins, only NS3 seems to affect control of p21 protein expression in a long-term *in vivo* infection. Nevertheless, the current studies indicate that neither expression of p21 protein nor that of viral NS3 protein can serve as a marker of progression of chronic hepatitis C to HCC *in vivo*. Definition of accurate relationships between NS3 protein and cell cycle control in *in vivo* conditions requires further studies.

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