Immunohistochemical Study of Cell Proliferation in Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC), the most common primary liver cancer, with a poor prognosis, is the fifth most common cancer in men and the seventh in women. The study was made on 32 surgically removed liver carcinomas. In order to compare results, we included a group of non-tumor lesions obtained by liver biopsy. Assessment of the proliferative activity of the studied liver lesions was made using immunohistochemical stains with the monoclonal Ki-67 antibody, clone MIB-1 ready-to-use (DAKO Cytomation CA, USA), in the LSAB-HRP work system. To appreciate the proliferation index of Ki-67 (PI Ki-67), we used the semi-automated method of counting the nuclei on digital images. The statistical analysis was performed using SPSS software, Version 20.0 (IBM SPSS Statistics) and Microsoft Office Excel 2007. Mean value of Ki-67 index was 0.4 ± 0.2 in normal liver, 3.52 ± 0.2 in non-tumor liver lesions and 13.4±7.7 in HCC ($p < 0.001$). In chronic hepatitis, PI Ki-67 varied between 2.5 and 5.8 %, with a mean value of 5.2% in portal chronic hepatitis and 5.5% in active chronic hepatitis with cirrhotic evolution. In HCC, the values of Ki-67 index were between 0.7% and 52%, with a mean of PI Ki-67 of 13.43±7.7. 66.6% of HCC associated with hepatitis B virus infection and those developped from a cirrhotic lesion had a high Ki-67 score ($p = 0.1$). The results we obtained showed: a low Ki-67 score in patients with well-differentiated HCC (G1) ($p<0.001$), with or without capsule infiltration ($p = 0.003$); high PI Ki-67 in 33.33% of HCC detected in the right hepatic lobe and those extended bilaterally at the moment of diagnosis ($p = 0.142$) and a significant relationship between high Ki-67 score and vascular invasion ($p < 0.001$), the presence of intrahepatic metastasis being correlated with a high proliferative rate ($p < 0.001$). Differences between the proliferation rate of HCC and non-tumor liver lesions ($p < 0.001$) show that the uncontrolled division of tumor cells can play an important role in the developpment of HCC.

Keywords: hepatocellular carcinoma, proliferation index of Ki-67, immunohistochemistry

Hepatocellular carcinoma (HCC), the most common primary liver cancer, with a poor prognosis, is the fifth most common cancer in men and the seventh in women. HCC is also the second leading cause of cancer-related mortality in the world [1, 2].

Ki-67 is the most frequently used marker associated with cell proliferation, its expression being associated with the prognosis of patients with HCC [3]. A thorough analysis of the cell cycle showed that the Ki-67 antigen is expressed during the G1, S and G1-M phases, but not during the G0 phase of the cell cycle [4]. Other authors consider that Ki-67 immunoreaction is closely related to the rate of tumor growth, an independent prognostic indicative of general and disease free survival rates [5].

After analyzing the statement that hepatocyte proliferation represents an adaptation answer to hepatic injuries [6] and the hypothesis formulated by Freeman A. et al [7] that hepatitis C virus (HCV) can induce direct hepatocyte proliferation (leading to cirrhosis and HCC), we assessed the immunohistochemical (IHC) expression of Ki-67 antigen using the monoclonal antibody MIB-1.

The aim of our study was to assess the proliferative activity of neoplastic and non-neoplastic hepatocytes, to identify potential correlations between histopathological features of HCC and its proliferative rate and to establish the role of Ki-67 expression as a possible prognostic factor in patients with radical resection of HCC.

Experimental part

Material and method

Our study included a number of 32 cases, selected from the casuistry of the Department of Pathology (DP) of the Clinical County Hospital Timi°oara, the patients being diagnosed and treated surgically for HCC.

The final processing of resection pieces was made by 10% formalin fixation for 24 h and paraffin inclusion

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following the classic technique and existent protocols (rinsing, dehydration, clearing, inclusion). Routine morphological investigation was made on sections stained with haematoxilin-eosin (HE), using the standard technique. Sections 5µ-thick were cut from paraffin blocks, stained HE and morphologically evaluated.

For the IHC study, we selected one block from each case and additional 5 µm thick sections were cut and mounted on Superfrost slides or silanized, to make sure that they do not detach during the pretreatment processes. To assess the proliferative activity, Ki-67 immunostaining was made on 32 hepatic carcinomas (and the surrounding hepatic tissue); for result comparison, we included a control group consisting of sections from normal hepatic tissue and hepatic biopsies diagnosed as: chronic hepatitis with C virus (24 cases), chronic hepatitis with B virus (6 cases) and hepatic cirrhosis (4 cases). For the external positive control of the reaction, we included in our study a tonsil frgment, while for the internal positive control we used the positive reaction of portal lymphocytes.

We used the monoclonal Ki-67 antibody, clone MIB-1 ready-to-use (DAKO Cytomation CA, USA) in the LSAB-HRP (labeled streptavidin biotin-horseradish peroxidase) work system. Sections were deparafinized and dehydrated. In order to block the endogenous peroxidase, sections were treated with 3% hydrogen peroxide (H_2O_2) for 5 min, while for antigen retrieval, sections were pretreated with microwaves in target retrieval solution at *p*H = 6 (Dako).

After a 30 min incubation with the primary antibody, followed by vizualisation of the reaction with the 3,3'-diaminobenzidine dyhidrochloride (DAB) chromogen for 10 minutes and counter-staining of the nuclei with haematoxilin, we obtained a dotted nuclear staining pattern, granular or homogenous, the reaction being considered as positive for any nuclear staining, regardless of its intensity.

Determination of *Ki-67 proliferation index* (PI Ki-67) was accomplished using the Nikon Eclipse i80 microscope, which has a digital vizualisation system for high resolution images. We calculated PI Ki-67 using the *semi-automated counting method* on digital images at x40 magnification, taking into consideration all current recommendations about the required minimum number of tumor cells to be quantified.

The semi-automated method used for counting the nuclei on digital images comprises the next steps: (1) tissue fragments immunostained with MIB-1 antibody are initially examined at magnification 10× to identify tumor areas with maximum nuclear density; (2) then, these are examined at 40× magnification and the nuclei are counted. This step was made using the morphometry software with a special Count function that allows accurate assessment of the nuclei. So, (3) the mouse is positioned on one nucleus at a time (of immunostained tumor cells – brown staining) and a marker is positioned on each positive nucleus (a green star); at the same time, the number of counted and marked nuclei is entered in a table (generated by the morphometry software). After the counting of positive (brown) nuclei, (4) the negative tumor nuclei are quantified using the same procedure, their number being automatically registered in the table, together with the stained ones; in this manner, (5) the value of PI Ki-67 on the evaluated digital image can be assessed. For each case, enough digital images are examined in order to attain the necessary number of tumor nuclei according to current recommendations.

PI Ki-67 assessment method allows exact identification of positive tumor nuclei, not including in the count other possible positive cells. In the case of tissue sections with elevated cell density, where nuclear superposition can appear, this counting method possesses a very high accuracy, with reduced inter-observer variability. For each digital image, Ki-67 index value was obtained by determining the percentage of positive cells from the total number of counted cells.

To accomplish this study, we consulted the records, databases of DP and files of the respective patients. All patients signed the inform consent according to the Helsinki Declaration.

Statistical Analysis

The Kolmogorov-Smirnov test was used for testing the distribution of numerical variables. Qualitative variables were presented as numbers and percentages. Parametric tests (t-test, ANOVA) were used for the assessment of differences between numerical variables with normal distribution and nonparametric tests (Mann–Whitney or Kruskal–Wallis tests) for variables with abnormal distribution. Chi-square ($\chi 2$) test (with Yates' correction for continuity) was used for comparing proportions expressed as percentages (*n* designates the total number of patients included in a particular subgroup). 95% confidence intervals were calculated for each predictive test and a p-value <0.05 was considered as significant for all statistical tests. The statistical analysis was performed using SPSS software, Version 20.0 (IBM SPSS Statistics) and Microsoft Office Excel 2007.

Results and discussions

The characteristics of the patients in the study group are presented in table 1.

Table 1
CHARACTERISTICS OF PATIENTS IN THE STUDY GROUP

	n (%)
Gender	
-Male	12 (37.5%)
-Female	20 (62.5%)
Age	
≤60	20 (62.5%)
>60	12 (37.5%)
Tumor localization	
-right hepatic lobe	12 (37.5%)
-left hepatic lobe	8 (25%)
-bilateral	12 (37.5%)
Associated pathology	
-cirrhosis	12 (37.5%)
-hepatitis B virus	12 (37.5%)
-hepatitis C virus	4 (12.5%)
Tumor size	
<5 cm	4 (12.5%)
>5 cm	28 (87.5%)
Histological type	
-trabecular	11 (34.3%)
-acinar	8 (25%)
-solid/ pelioid	6 (17.7%)
-clear cells	5 (15.6%)
-anaplastic	2 (6.2%)
Tumor differentiation	
G1	6 (18.7%)
G2	24 (75%)
G3-4	2 (12.5%)

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The incidence of Ki-67 expression on the studied patients is presented in table 3.

Table 2

CHARACTERISTICS OF PATIENTS IN THE CONTROL GROUP

	n (%), mean±standard deviation
Gender -Male -Female	 25 (65.7%) 13 (34.3%)
Age (years)	39.9±2.1
Ki-67 expression -positive -negative	 21 (55.2%) 17 (44.8%)

In the non-tumor lesions, we correlated the Ki-67 expression with the epidemiological parameters (gender and age), liver transaminases and with the histopathological characteristics (interface hepatitis, portal space inflammation, necroinflamatory activity score and fibrosis) (table 4).

Table 3

INCIDENCE OF POSITIVE Ki-67 EXPRESION IN STUDIED PATIENTS

	Positive Ki-67	IP Ki-67	Negative Ki-67
Normal liver	1/4 (25%)	0.4%	3/4 (75%)
Hepatitis B virus	3/6 (50%)	3 % (2.5-3.5%)	3/6 (50%)
Hepatitis C virus	15/24 (62.5%)	5.2 % (2-5.8%)	9/24 (27.5%)
Cirrosis	2/4 (50%)	5.5 % (5.1-6.1%)	2/4 (50%)

Mean value of PI Ki-67 varies between 0.4% in normal liver tissue and 5.5 % in active chronic hepatitis (ACH) with cirrhotic evolution. We noted rare reactive Ki-67 nuclei scattered in normal hepatocytes, with a PI Ki-67 of 0.4%. In chronic hepatitis, we found a large variation of PI Ki-67, with vaues between 2.5 % and 5.8 % and a mean value of 5.2 % in portal chronic hepatitis (PCH) and 5.5 % in ACH with cirrhotic evolution (fig. 1).

The association between Ki-67 expression and characteristics of patients are presented in table 5.

Table 4

Ki-67 RELATIOSHIP WITH NON-TUMOR LIVER PATIENTS CHARACTERISTICS

	Positive Ki-67 (n = 21)	Negative Ki-67 (n = 17)	P value
Gender (M/F)	15/6	10/7	0.6
Mean age (years)	41.2	38.7	0.3
AST (UI/l)	209	168	<0.001
ALT	130	133	0.7
Necroinflammation score	9.8	8.2	0.02
Portal inflammation score	3.1	1.5	0.04
Fibrosis score	1.8	2.1	0.1
Steatosis score	17	5	0.004

ALT-alanin-aminotransferase; AST-aspartat-aminotransferase.

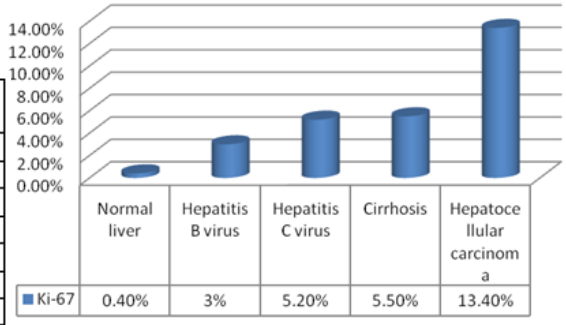

Fig. 1. Ki-67 values in different liver lessions

	No. of cases n (%)	High Ki-67 score	Low Ki-67 score	P value
Gender -Male -Female	 12 (37.5%) 20 (62.5%)	 8 (66.6%) 4 (20%)	 4 (33.3%) 16 (80%)	 0.1 0.003
Age ≤60 >60	 20 (62.5%) 12 (37.5%)	 8 (40%) 4 (33.3%)	 12 (60%) 8 (66.6%)	 0.4 0.1
Tumor localization -right hepatic lobe -left hepatic lobe -bilateral	 12 (37.5%) 8 (25%) 12 (37.5%)	 4 (33.3%) 4 (50%) 4 (33.3%)	 8 (66.6%) 4 (50%) 8 (66.6%)	 0.1 0.7 0.1
Associated pathology -cirrhosis -hepatitis B virus -hepatitis C virus	12 (37.5%) 12 (37.5%) 4 (12.5%)	 8 (66.6%) 8 (66.6%) 0 (0%)	 4 (33.3%) 4 (33.3%) 4 (100%)	 0.1 0.1 <0.001
Tumor size <5 cm >5 cm	 4 (12.5%) 28 (87.5%)	 4 (100%) 8 (28.5%)	 0 (0%) 20 (71.4%)	 <0.001 0.04
Histological type -trabecular -acinar -solid/ pelioid -clear cells -anaplastic	 11 (34.37%) 8 (25%) 6 (17.75%) 5 (15.63%) 2 (6.25%)	 7 (63.6%) 2 (25%) 3 (50%) 0 (0%) 0 (0%)	 4 (36.3%) 6 (75%) 3 (50%) 5(100%) 2 (100%)	 0.2 0.017 0.715 <0.001 <0.001
Tumor differentiation G1 G2 G3-4	 6 (18.75%) 24 (75%) 2 (12.5%)	 0 (0%) 12 (50%) 0 (0%)	 6 (100%) 12 (50%) 2 (100%)	 <0.001 0.7 <0.001
Capsule formation -Present -Absent	 20 (62.5%) 12 (37.5%)	 4 (20%) 8 (66.6%)	 16 (80%) 4 (33.3%)	 0.003 0.1
Capsule infiltration -Present -Absent	 20 (62.5%) 12 (37.5%)	 4 (20%) 8 (66.6%)	 16(80%) 4 (33.3%)	 0.003 0.1
Vascular invasion -Present -Absent	 24 (75%) 8 (25%)	 20 (83.3%) 8 (100%)	 4 (16.6%) 0 (0%)	 <0.001
Hepatic metastasis -Present -Absent	 16 (50%) 16 (50%)	 16 (100%) 12 (75%)	 0 (0%) 4 (25%)	 <0.001 0.01

Table 5

ASSOCIATION BETWEEN Ki-67 AND CHARACTERISTICS OF PATIENTS

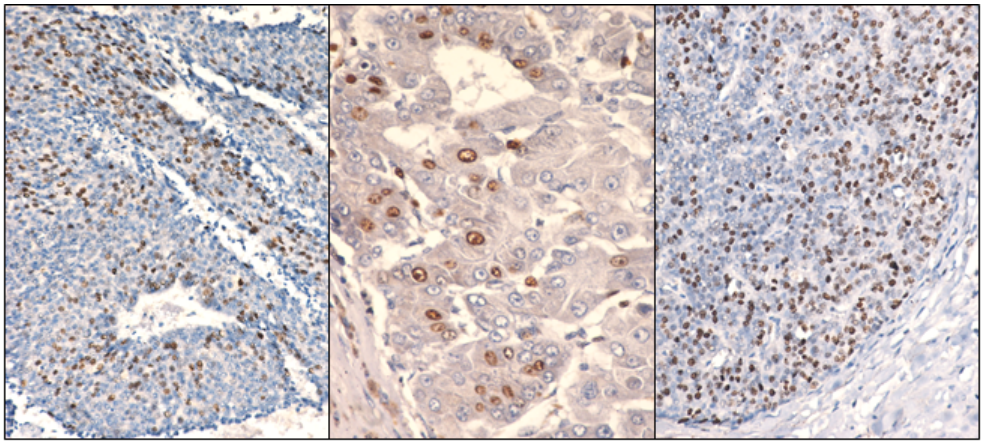

Fig. 2. Ki-67 expression in liver tumors: a. trabecular/sinusoidal; b. acinar; c. solid HCC

Mean value of Ki-67 index was 0.4±0.2 in normal liver, 3.52±0.2 in non-tumor liver lesions and 13.4±7.7 in HCC.

We found significant statistical differences between them, $p < 0.001$.

In HCC we found values of Ki-67 index between 0.7% and 52 %, with a PI Ki-67 mean value of 13.43±7.7. We divided them into two groups: HCC with high PI Ki-67 (≥15%) - 12 cases (37.5%) and HCC with low PI Ki-67 (<15%)- 20 cases (62.5%).

We found a higher frequency of HCC with high PI Ki-67 in male gender ($p = 0.1$), while in women, HCC with low PI Ki-67 prevailed (80% cases), with significant statistical differences ($p = 0.003$). In patients older then 60 years, the majority was made of tumors with low Ki-67 index (66.6% cases) ($p = 0.1$).

We didn't find any association between the Ki-67 score and tumor localization. The results we obtained showed: a low Ki-67 score in patients with well-differentiated HCC (G1) ($p < 0.001$), with or without capsule infiltration ($p = 0.003$); high PI Ki-67 in 33.33% of HCC detected in the right hepatic lobe and those extended bilaterally at the moment of diagnosis ($p = 0.142$) and a significant relationship between high Ki-67 score and vascular invasion ($p < 0.001$), the presence of intrahepatic metastasis being correlated with a high proliferative rate ($p < 0.001$).

Cell proliferation markers were used to assess the prognosis of various tumors and seem to represent promising prognostic factors in HCC [8]. Proliferation index can be determined IHC using the monoclonal antibody Ki-67 (clone MIB-1), which reacts with the nuclear protein Ki-67. The expression of Ki-67 was identified as an independent predictor of rapid tumor recurrence in patients after hepatic transplant [9] and as a marker of poor prognosis after early tumor recurrence [10]. However, Ki-67 positive expression did not correlate with the outcome of the disease in various studies [11, 12], but correlated independently with the level of serum transaminase and the viral etiology of hepatic disease. Most HCC appear in cirrhotic livers after years of chronic liver inflammation caused by hepatitis viral infection, alcoholic and non-alcoholic steatohepatitis [13, 14].

Using the monoclonal antibody Ki-67 (clone MIB-1), we investigated the IHC expression of Ki-67 antigen on tissue sections fixed in formalin and included in paraffin, obtained by surgical resection from 32 patients with HCC and we assessed its relationship with tumor features. On a control group made up of 34 hepatic non-tumor lesions (chronic hepatitis with HCV -24 cases, chronic hepatitis with HBV - 6 cases and hepatic cirrhosis -4 cases), we evaluated the expression of Ki-67, identifying Ki-67 positive hepatocytes, on samples obtained by liver biopsy.

In non-tumor hepatic lesions, we observed positive Ki-67 immunoreaction in 55.02% of examined cases, staining of sections with MIB-1 highlighting a nuclear pattern. PI Ki-67 varied between 0.4% in normal liver tissue and 6.1% in hepatic cirrhosis. We obtained a mean value of PI Ki-67 of 3.0% in PCH (between 2.5-3.5%), 5.2% in ACH (between 2.0-5.8%) and 5.5% in hepatic cirrhosis (between 5.1-6.1%). Assessing the parameters associated with age, sex and serum transaminase levels in the two groups of patients (Ki-67 positive and negative), we noted the absence of a clear relationship between the positive Ki-67 expression and age ($p = 0.311$) or sex of the patients ($p = 0.638$) and a slightly elevated level of serum transminase for the group of Ki-67 positive patients (ALT 209 UI/l vs. 168 UI/l) ($p < 0.001$).

Taking into account the statement of Canchis WP et al [6] that the relationship between hepatocyte proliferation and necroinflammation, fibrosis score and serum level of alkaline phosphatase in chronic hepatitis with C virus is not well-known, we assessed some histopathological aspects. We noted, in agreement with the observations of Freeman A et al. [7], an association between the percentage of Ki-67 positive hepatocytes and interface hepatitis, portal inflammation and lobular inflammation. Necroinflammatory activity score and portal inflammation score were slightly, but significantly elevated in Ki-67 positive patients, as compared with Ki-67 negative patients (9.8 vs. 8.2; $p = 0.028$ and 3.1 vs. 1.5, respectively; $p = 0.043$); we did not observe any relationship between fibrosis score and Ki-67 expression ($p = 0.199$). Instead, we found association of steatosis, more frequently in Ki-67 positive patients (80.95%) than in negative ones (29.41%) ($p = 0.004$).

In agreement with the results of Canchis WP et al. [6], in patients with HCV we noted an elevated proliferative activity, parallel with the progression of histological lesions in liver disease.

Assessing the proliferative rate on 91 liver biopsies from patients with chronic hepatitis, liver cirrhosis, regenerative nodules and hepatic cirrhosis with cancer, Faciolli S et al [15] observe in chronic hepatitis and cirrhosis a mean PI Ki-67 of 16%, close to that of malignant tumors, recording a PI Ki-67 of 20% in high grade HCC and the absence of Ki-67 expression in many low grade HCC. Correlating PI Ki-67 with patient survival, the authors do not register a better prognosis for HCC with low PI Ki-67.

Based on the results obtained after assessing the proliferative rate in chronic hepatitis with HCV (34 cases), as compared with hepatic lesions of other etiology, Farinatti F et al. [16] show that HCV infection induces an abnormal proliferation of hepatocytes, the latter being associated with an elevated risk of hepatic cancer in patients with HCV infection.

We obtained a PI Ki-67 mean value of 0.4±0.26% in normal liver, 3.52±0.23% in non-tumor benign lesions and 13.43±7.7% in HCC, the differences between HCC and non-neoplastic liver tissues being statistically significant ($p < 0.001$). The studied HCC presented PI Ki-67 values between 0.7 and 52.0%, with a mean of 13.43±7.7% and were divided into two groups: (a) HCC with high PI Ki-67 (≥ 15%) – 12 cases, (37.5%) and (b) HCC with low PI Ki-67 (<15%) – 20 cases (62.5%).

We analyzed the relationship between PI Ki-67 and clinical-pathological factors: sex and age of the patients, localization of tumor and associated pathology, size of the tumor, histological type, degree of tumor differentiation, presence/absence of the capsule and vascular invasion.

In male patients, we observed a higher frequency of HCC with high PI-Ki-67 ($p = 0.1$), while in female patients HCC with low PI Ki-67 prevailed (80%) ($p = 0.003$).

We did not find any relationship between the Ki-67 score and tumor localization ($p=0.142$). At the moment of diagnosis, 33.33% of HCC from the right hepatic lobe and those extended bilaterally proved to be aggressive tumors with high PI Ki-67 ($p = 0.142$). Half (50%) of carcinomas in the left hepatic lobe presented high values of PI Ki-67 ($p = 0.715$).

Assessing the relationship between Ki-67 expression and associated pathology, we noted high PI Ki-67 values in 66.67% of HCC associated with HBV and those developed on cirrhotic tissues ($p = 0.143$) and a low Ki-67 score ($p < 0.001$) in all HCC associated with HCV.

All tumors <5cm and 28.57% of HCC ≥ 5 cm presented a high Ki-67 score ($p < 0.001$); 71.43% of HCC ≥ 5 cm had PI Ki-67 under the mean value ($p = 0.046$).

We observed no relationship between the Ki-67 score and the histological type. HCC with trabecular and solid/pelioid pattern were associated with high values of PI Ki-67 -in 63.64% ($p = 0.258$) and 50% of cases ($p = 0.715$), respectively.

We noted a clear relationship between low grade HCC (G1) and low Ki-67 score (PI Ki-67 6.8%) ($p < 0.001$); 50% of HCC moderately differentiated presented a high Ki-67 score (PI Ki-67 31.46%).

The results we obtained show a significant relationship between low Ki-67 score and HCC with or without capsule infiltration ($p = 0.003$), between high Ki-67 score and vascular invasion ($p < 0.001$), the latter being present in 83.33% of HCC with high PI Ki-67 and 16.67% of those with low PI Ki-67. All HCC with intrahepatic metastases ($p < 0.001$) and 75% of HCC without metastases ($p = 0.017$) had a high Ki-67 score, the presence of intrahepatic metastases being thus correlated with the Ki-67 index.

PI Ki-67 is considered an objective marker that reflects the proliferative activity of hepatocytes [17], the intensity of Ki-67 expression being correlated with the prognosis of HCC [18, 19].

Recurrence is the main factor that influences the prognosis of hepatic cancer after tumor resection, the rate of HCC recurrence after resection being disappointingly high [20]. It has been shown that HCC with high Ki-67 expression presented recurrences within the first year after tumor resection. Patient prognosis assessment by quantitative analysis on liver biopsies stained IHC before treatment could offer the basis for accurate therapy selection in patients with HCC.

The results of the study conducted by King KL et al. [3] on 67 patients with HCC surgically resected suggest that Ki-67 expression is an independent prognostic factor for patients with HCC after resection and can be useful in choosing adjuvant therapies. In a more recent study that evaluated Ki-67 expression in HCC and its relationship with prognosis, Shi W et al. [21] describes the relationship between Ki-67 expression and prognosis, considering Ki-67 as an independent prognosis factor for patients with HCC.

The prognosis of HCC after hepatic resection depends on 3 main factors: histological features of HCC, extension and characteristics of the tumor and the type of surgical treatment. A lot of studies showed that oncogenes play an important role in the development, progression and metastasis of solid tumors, various oncogenic factors being recently identified.

Conclusions

Ki-67 is a useful marker for assessing the proliferative activity of normal and neoplastic hepatocytes.

Differences between the proliferative rate of HCC and that of non-neoplastic hepatic lesions ($p < 0.001$) show that uncontrolled cell division can play an important role in the development of HCC.

Ki-67 expression as a prognostic marker can represent a criterion in the selection of appropriate therapy for HCC and a future target for molecular therapy.

To prove the prognostic value of Ki-67 in patients with HCC, studies on an adequate number of patients, with standardized treatment procedures are required.

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