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ARTICLE

Immunohistochemical Assessment and Prognostic Value of Hepatitis B Virus X Protein in Chronic Hepatitis and Primary Hepatocellular Carcinomas using *anti-HBxAg* Monoclonal Antibody

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Hepatitis B virus (HBV) is the most meaningful risk factor in chronic hepatitis, cirrhosis and primary hepatocellular carcinoma (PHC). The hepatitis B virus X protein (HBxAg) is a multifunctional protein with many important functions in hepatocellular carcinogenesis. A monoclonal anti-HBxAg antibody was developed in our laboratory and characterized by different methods. Using this antibody HBxAg was detected in formaldehyde fixed paraffin embedded tissue sections of 72 liver biopsies from patients with acute hepatitis, chronic hepatitis, cirrhosis and primary hepatocellular carcinoma. The co-expression of hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg) and HBxAg was compared. The histological and cytological localization of the detected HBxAg showed a characteristic distribution in different stages of HBV infection. Strong and diffuse nuclear reaction was detected in PHC cases in contrast to the focal, cytoplasmic and nuclear labeling in the acute and chronic B hepatitis cases. Our antibody seems to be a suitable prognostic marker for routine pathohistological diagnosis and for comparative pathological and epidemiological research on the development of PHC. (Pathology Oncology Research Vol 7, No 3, 178–184, 2001)

Keywords: primary hepatocellular carcinoma, hepatitis-B X protein, chronic B virus hepatitis, monoclonal anti-HBx antibody, immunohistochemistry

Introduction

Hepatitis B virus (HBV) is one of the main etiologic factors in the development of chronic B hepatitis, liver cirrhosis, and primary hepatocellular carcinoma (PHC).[11] The World Health Organization estimated that 400 million people were chronically infected with HBV virus in the year 2000.[1,25] Approximately one million people suffering from HBV-related chronic hepatitis or PHC die each year.[27] The relative risk of HBV carriers to develop PHC approaches 200 to 1, which is one of the highest relative risk known for a human cancer.[12]

Hepatitis B X antigen (HBxAg) is the product of the fourth and smallest open reading frame (X ORF) expressed during the life cycle of HBV.[17,24] The X gene is conserved among mammalian hepadnaviruses and its product the X protein (HBxAg) is a multifunctional regulatory molecule which, although does not directly bind to DNA,[1] modulates many host functions[27] involved in cell proliferation, house-keeping functions, cytokine networks and the acute immune response.[27,38,42]

Our primary goal was to develop monoclonal antibodies against the X protein, because immunological detection of HBxAg in different stages of HBV infection related diseases would be important in diagnosis and prognosis. There are no commonly used immunoserological and

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immunohistochemical detection kits for HBxAg available yet in the routine laboratory or in research to investigate the risk for the development of PHC in HBV infected patients. Our further aim was to compare the histochemical results of our monoclonal antibody with other HBV antigen specific antibodies, and with the literature data. We planned a retrospective immunohistochemical study on a representative group of clinically verified HBV infected patients.

A monoclonal antibody family against HBxAg was developed and characterized in our laboratory for research and diagnostic purposes. To examine the pathogenesis of PHC our mAb has been used for immunohistological staining of formaldehyde fixed and paraffin embedded tissue specimens of liver biopsies. Based on the histological and cytological appearance of the immunostaining with HBxAg specific monoclonal antibody, the prognostic efficacy was investigated.

Materials and Methods

Antigen

Two constructs of recombinant HBxAg were used (Marczinovits et al.[26]) for monoclonal antibody and assay development. The hepatitis B virus X gene (HBx) was cloned into the fusion expression vector pRIT2T or into pGEX-3X, resulting a HBx-staphylococcal protein A (HBxAg-pA) or a HBx-GST (HBxAg-GST) fusion gene construct, respectively. Both constructs were expressed in *E.coli* DH5. HBxAg-pA was used for immunization in crude form. Affinity purified HBxAg-GST was used for testing the antibodies.

Hybridoma development

Female inbred (bred and kept under SPF conditions) BALB/c mice (Charles River Inc., USA) were immunized repeatedly. The splenocytes of the most responding animal were fused to Sp-2/0-Ag14 (ATTC, USA) mouse myeloma cells according to the method described by Köhler and Milstein (1975).[21] Hybrids were selected on HAT containing culture medium (DMEM, Gibco, USA).

Immunoserological characterization by ELISA

Cell culture supernatants were first tested by simple-binding ELISA described by Engvall et Perlman.[6] HBxAg-GST was used as antigen for screening to exclude cross-reactions with the carrier proteins used for immunization.

Microtitre plates (Dynatech, USA) were sensitized with 5 μg/ml of recombinant HBxAg in 0.1M bicarbonate buffer, at pH 9,6 overnight at 4°C, PBS-gelatin (0,5%) (Sigma, USA) was used to saturate the free binding sites. The hybridoma supernatants were tested in different dilutions. Reactions were developed by HRPO labeled rabbit anti-mouse Ig (Dakopats, Denmark) and measured by microphotometer (Dynatech MR 7000, USA) at 490 nm wavelength.

The isotype subclasses of the mAbs were determined using a mouse isotype kit (Sigma Chem. Co., USA)

Immunoblot determination

The HBxAg-GST and HBxAg-pA fusion proteins and the glutathione S-transferase (GST) enzyme protein were used for SDS-PAGE electrophoresis (Laemmli, 1970).[23] The samples were run on a 15% polyacrylamide gel and stained with Coomassie blue. Proteins were electrophoretically transferred from the gel to a nitrocellulose paper using a semidry system according to Towbin et al. (1979).[37] The free binding sites of nitrocellulose were saturated with 5% nonfat dried milk (PBS-milk) for 2 hours at room temperature. The membrane was washed three times in washing buffer for 30 min and the hybridoma supernatants were added (1:1000) for 2 hours at room temperature. The samples were washed three times and then incubated with HRPO conjugated goat anti-mouse IgG (Dakopats, Denmark) for 1 hour at room temperature. The reactions were visualized by diaminobenzidine tetrahydrochloride (DAB, Sigma Chem Co. USA) as substrate.

Immunohistochemical characterization

The specificity of monoclonal anti-HBxAg antibody clones was analyzed in different human tissues. Formaldehyde fixed and paraffin embedded tissue sections from clinically verified B hepatitis positive liver biopsies were used as positive controls. HBV negative, normal and pathologic tissues originating from the tissue bank of Histopathology Ltd. were analyzed during the further immunohistochemical characterization of monoclonal anti-HBxAg clones. An IgG2a (anti-CD45RO, clone UCHL-1; Immunotech Inc., France) monoclonal antibody was used as irrelevant, negative control during the whole immunohistochemical investigations.

Retrospective immunohistochemical study

A retrospective study on needle biopsy specimens from patients with acute viral hepatitis (10), chronic hepatitis (45), liver cirrhosis (9) and surgical biopsy specimens from patients with PHC (8) was performed after the selection of the best anti-HBxAg clone (No. 3F6/G10 with IgG2a isotype). The tissue samples were obtained from the Pathology Service of the County Hospital of Baranya in Pécs, County Hospital of Zala in Zalaegerszeg and from the Histopathology Ltd., Pécs. Formaldehyde-fixed, paraffin

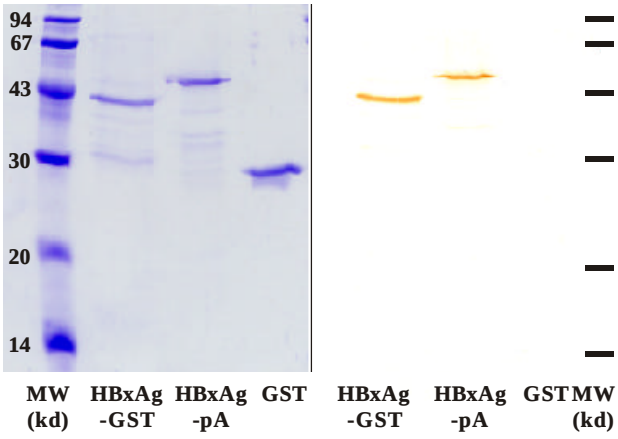

Figure 1. The HBxAg SDS-polyacrylamide gel electrophoresis (left) and immunoblot (right) developed by using anti-HBxAg (clone: 3F6/G10) IgG2a monoclonal antibody.

embedded sections were stained by polyclonal (goat) biotinilated anti-HBsAg (Dacopatts, Denmark) and biotinilated (rabbit) anti-HBcAg (Dakopatts, Denmark) antibodies. The findings were compared to the results of immunohistochemical investigations by monoclonal anti-HBxAg (IgG2a) antibody. Biotin labeled anti-mouse Ig (Dakopatts, Denmark) was used as secondary antibody in the case of anti-HBxAg. The reactions were developed by the HRPO streptavidin–biotin complex method. Immunostaining was visualized by H_2O_2-amino ethyl carbasol (Sigma Chem. Co., USA) substrate and the sections were then counterstained with hematoxylin. Irrelevant monoclonal antibodies as the anti-FITC $IgG1^2$ and the anti-UCHL-1 IgG2a (Immunotech Inc., France) were used as negative controls.

Results

An anti-HBxAg monoclonal antibody family was developed following the usual way against recombinant hepatitis B virus X protein (the development of recombinant antigen we published previously[26]). Monoclones were selected and characterized immunoserologically and immunohistochemically. Monoclonal IgG2a antibody secreted by hybridoma clone 3F6/G10 was alike optimal for ELISA, immunoblot and immunocytochemistry. The anti-HBxAg monoclonal antibody recognized (in 5 ng/sample dilution) the recombinant antigen (in 250 ng/sample) as proved by high optical density (OD490 ≥ 1.000) measured in a simple binding ELISA. 2.3 ng/well antigen concentration (calculated for the X protein) was the minimum antigen level in the optimal measurable range (OD490 ≥ 0.400). No reactions were found with the fusion partners (GST, and Protein A) or other non-specific mammalian and non-mammalian proteins. (Data not shown.)

Further characterization of anti-HBxAg antibody clone by immunoblot technique showed obvious positivity in the relevant band *(Figure 1).*

The immunohistochemical characterization did not show any positive reaction on different normal human tissues (embryonic and adult) and non-mammalian samples. However, intensive immune reactivity was found in liver biopsy samples from chronic hepatitis B virus carrier patients in different histological and cellular localization. Granular cytoplasmic and nuclear stainings were present in equal density in some cases and an exclusively intense nuclear positivity in some others *(Figure 2).*

Retrospective histopathological analysis was performed on 72 liver biopsy samples of clinically verified hepatitis B virus holding patients. We analyzed the microscopic appearance and the type of intracellular localization of HBxAg immunostaining according to the classification of commonly accepted literature data.[41]

The highest number of HBxAg positive cases was found in the group of patients with chronic hepatitis (86.6%) in heterogeneous microscopic appearances. Between 40 to 50% of the biopsy samples showed HBxAg positivity in the groups of acute hepatitis, liver cirrhosis and primary hepatocellular carcinoma. The histologic and cytologic distribution of HBxAg showed various forms in each group. The frequency of positive cells was the highest in chronic hepatitis and PHC, however, the intensity of the immune reactions was ambiguously strongest in the group of PHC samples.

Intracellular localization after anti-HBxAg immunostaining showed cytoplasmic and/or nuclear distributions in general, but dominant nuclear positivity was found in PHC. The histological occurrence of labeling with anti-HBxAg monoclonal antibody showed a focal pattern (localized in well defined groups of HBxAg positive cells between broad negative regions, marked as "L" in *Table 1.*) in the majority of acute and chronic B hepatitis biopsy samples. Moderate cell membrane staining was found in one chronic B hepatitis case with granular cytoplasmic and heterogeneous nuclear labeling *(Figure 2a).* The cytoplasmic occurrence was granular in some cases, fine granular (diffuse) in the other samples independently from the clinical and histopathologic stages of chronic hepatitis or cirrhosis. *(Figure 2a and 2b).* A predominantly nuclear appearance was typical with diffuse histologic pattern in a few chronic hepatitis samples *(Figure 2c).*

We found characteristically intense and uniform nuclear immunoreactions with HBxAg antibody in each cell of the entire liver tissue (both in the tumor and in the residual liver) on PHC cases with moderate cytoplasmic labeling *(Figure 2d and 2e).* The results of the anti-HBxAg retrospective study on 72 biopsy samples with detailed data of the histologic and cytologic microscopical appearance are summarized in *Table 1.*

To exclude non-specific nuclear reactions, which could occur in some formaldehyde fixed samples using mouse antibodies, we tested all tissue samples with an irrelevant

mouse monoclonal antibody as negative control (anti-UCHL-1 IgG2a) but no false positive immunoreactivity was found.

We compared the microscopic immunostaining pattern of monoclonal anti-HBxAg antibody with two commercially available polyclonal antisera against other hepatitis B virus antigens. In the laboratory daily routine the determination of both "s" (surface) and "c" (core) antigens are used for primary diagnosis and as prognostic factors. Our monoclonal antibody showed significantly more sensitive reactions in the group of chronic hepatitis patients than the HBsAg or HBcAg antibodies. The lim-

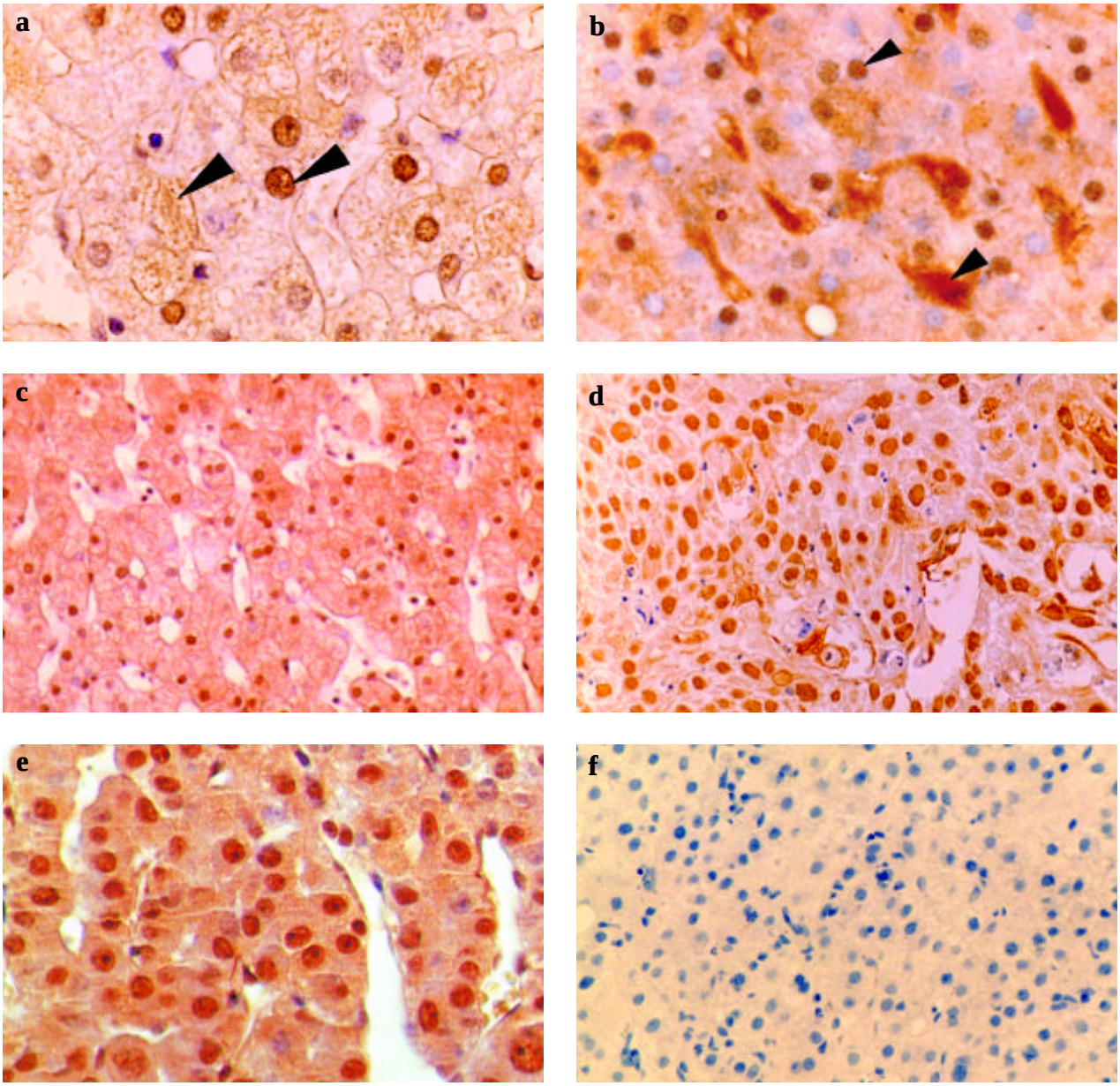

Figure 2. Immunhistochemical detection of the HBV X antigen in paraffin sections of liver biopsies from patients with HBV-associated chronic hepatitis and primary hepatocellular carcinoma stained by anti-HBxAg (clone: 3F6/G10) IgG2a monoclonal antibody. (a) Granular reaction in the cytoplasm with moderate membrane labeling and miscellaneous nuclear positivity (arrows) can be show on liver cells from a patient with chronic hepatitis. 400x (b) Strong diffuse and moderate granular reactions in the cytoplasm and various nuclear staining in chronic hepatitis. 400x (c) Intensive diffuse nuclear staining with moderate granular cytoplasmic labeling in chronic hepatitis. 100x (d) Primary hepatocellular carcinoma shows characteristically strong diffuse positive nuclear staining with moderate cytoplasmic reaction in all cells. 250x (e) Typically strong diffuse nuclear labeling in PHC at higher magnification. 400x (f) No immunoreactivity can be detected in a negative control (toxic hepatitis) stained by the same antibody under the same conditions as the previous samples. Hematoxilin counterstaining, 200x

Table 1. **Immunohistochemical analysis of HBxAg expression on 72 liver biopsy samples**

			Anti-HBxAg immunoreactivity											
			Positivity in cells (%)			*Intensity of staining*			*Intracellular localization*			*Occurrence in liver tissue*		
Diagnosis	*No.*	*Positive(%)*	*<30%*	*30-70%*	*>70%*	*+*	*++*	*+++*	*M*	*C*	*N*	*S*	*L*	*D*
Acute	10	4 (40)	4	–	–	–	4	–	–	4	4	–	4	–
Chronic	45	39 (86,6)	13	22	4	6	30	3	1	39	39	3	24	12
Cirrhosis	9	4 (44,4)	4	–	–	3	1	–	–	4	4	1	2	1
PHC	8	4 (50)	–	2	2	–	1	3	–	1	4	–	–	4

Acute: acute B virus hepatitis; *Chronic:* chronic B virus hepatitis; *Cirrhosis:* hepatitis B virus post infectious liver cirrhosis; *PHC:* primary hepatocellular carcinoma
M: membranous; *C:* cytoplasmic; *N:* nuclear; *S:* scattered; *L:* localized; *D:* diffuse;

ited number of the investigated cases in other groups did not allow correct calculations in liver cirrhosis and PHC, but the tendency is clear. See details in *Table 2. Table 3* summarizes the microscopic patterns of HBxAg positivity in different histopathological stages of HBV infection-related diseases.

Discussion

The role of HBxAg in the progression of chronic B hepatitis and the generation of PHC has only Geen understood for a decade. The presence of HBxAg in chronic B hepatitis and in primary hepatocellular carcinoma was detected by immunohistochemistry,[18,31,35,40,41] serological studies,[7,19,22,29] and reverse transcription-polymerase chain reaction (RT-PCR) on human biopsy samples,[4,30,36] transformed cell lines[28,32] and in a transgenic murine model[20]. There are remarkable differences in the current literature regarding the pathognomic role of HBxAg: we found detailed experimental results of current molecular and cellular biological techniques, but no summarized multi-centric clinico-pathological and epidemiological studies have get been published. Standardized immunological techniques are required for large-scale comparative serological and histochemical analyses. The development of such methods needs anti-HBxAg antibodies with high specificity and high affinity without any cross-reactions with other antigens.

The generation of polyclonal and monoclonal antibodies against HBxAg is technically difficult because of a special physico-chemical feature of this protein: 54% of the molecule is composed of hydrophobic amino acids. Conventional biochemical methods to isolate this protein proved to be inappropriate because the separated HBxAg protein precipitates in water.[13] The special secondary and tertiary molecular structure of HBxAg restricts all practical applications of antibodies developed against overlapping synthetic peptides constructed on the base of the primary sequence.[34] For this reason we used a recombinant antigen for monoclonal antibody production and assay development.[26] As a result of careful planning of the antigen structure our anti-HBxAg IgG2a antibody is useful in both immunoserological and immunohistochemical techniques.

The histochemical studies in literature on PHC and chronic B hepatitis samples were performed by less characterized (mostly polyclonal) antibodies.[35,40,41] These papers described the presence and microscopic occurrence of HBxAg in chronic hepatitis, liver cirrhosis, and PHC.[3,7,8,9,10,11,12,14,44] The distribution of the incidence and histopathologic appearance of HBxAg positive cases

Table 2. **Comparative immunohistochemical analysis of the expression of HBsAg, HBcAg, and HBxAg on a representative group of HBV related diseases**

		Positive		
Diagnosis	*No.*	*HBsAg*	*HBcAg*	*HBxAg*
Chronic	25	10 (40%)	5 (20%)	22 (88%)
Cirrhosis	9	1 (11%)	1 (11%)	2 (22%)
PHC	6	1 (17%)	1 (17%)	2 (33%)

HBsAg and HBcAg were detected by polyclonal antisera, HBxAg was detected by monoclonal antibody

Table 3. **Tendency of histological and cytological localization of HBxAg in different stages of HBV related diseases according to the data of immunohistochemical assessment on 72 liver biopsy samples**

	Histological appearance		*Cytological appearance*	
HBV related disease	*Focal*	*Diffuse*	*Cytoplasmic*	*Nuclear*
Acute hepatitis	+	–	+	+/–
Chronic B hepatitis	++	+	++	+
Liver cirrhosis	+	+	+/–	+
PHC	–	++	+/–	+++

between the research studies was more or less comparable, but depending upon the antibody used.[18,31,40,41]

Our monoclonal antibody allows us to precisely analyze the incidence of the HBxAg positivity and the microscopic localization on formol-paraffin biopsy samples in the context of a retrospective histopathological study.

During our retrospective study the incidence of HBxAg both in the chronic B hepatitis and the PHC groups was similar to the data of Zentgraf et al.,[43] Vitvitski-Trepo et al[39] and Su et al,[35] but it was significantly different from the data presented by Wang et al,[40,41] who found HBxAg positivity in over 80% of all groups of hepatitis B virus related diseases using polyclonal (rabbit) antibody developed against synthetic antigen. Seo et al[31] found similar incidence to ours in chronic B hepatitis and liver cirrhosis, but in the PHC cases their investigations detected HBxAg positivity in only 17%.

According to different reports, Haviv et al,[15] and Henkler et al,[16] found HBxAg exclusively in a nuclear localization. Perinuclear localization was observed by Sirma et al.[33] A predominantly cytoplasmic localization of the X antigen was observed by Zentgraf et al.[43] Both cytoplasmic and nuclear localization was described by Doria et. al[5] and Nomura et al.[28] HBxAg positivity was co-localized with proteosomes in the study of Sirma et al[33] and the association of HBxAg with mitochondria was described by Henkler et al.[16]

In *B hepatitis samples* both cytoplasmic and nuclear labeling was found by anti-HBxAg monoclonal antibody. In acute hepatitis we found focal labeling with dominantly cytoplasmic and moderate nuclear staining. However, major differences were detected in different stages of HBV infection: chronic B hepatitis samples showed strong diffuse (fine granular) or granular cytoplasmic and moderate nuclear positivity in focal pattern in the liver. Anti-HBxAg monoclonal antibody showed intense labeling in the nuclei and only moderate in the cytoplsams in liver cirrhosis samples. Remarkable cell membrane and granular cytoplasmic staining was found with a focal histological pattern in one chronic B hepatitis case.

In the *PHC cases* characteristically strong nuclear labeling was detected with diffuse appearance in the whole liver.

The core (HBcAg) and surface (HBsAg) antigen specific antibodies showed no similar tendency compared to anti-HBxAg antibody. Microscopic immunolabeling showed scattered light cytoplasmic and predominantly nuclear reactions without specific pictures of the stages of HBV related diseases by either anti-HBsAg or anti-HBcAg antibodies. Our immunohistochemical comparative analysis of the expression of HBsAg, HBcAg and HBxAg showed similar pattern to the results of PCR analysis published by Diamantis et al.[4] the expression of HBV "x" protein is significantly higher than the expression of "s" or "c" antigens. These results emphasize the patho-biological and technical advantage of the analysis of HBxAg in all HBV related diseases both in routine diagnostics and research investigations.

The HBxAg affects both cellular compartments simultaneously during the HBV infection: it activates mitogenic signaling cascades in the cytoplasm and interacts directly with transcription factors and with specific components of the basal transcriptional apparatus according to the literature data.[1]

The immunohistochemical analysis of histological and cytological localization of the HBxAg could be used as a patho-biological prognostic factor in the progression of the disease from the chronic hepatitis stage to the primary hepatocellular carcinoma. The diffuse and pre dominantly nuclear positivity of HBxAg in the entire liver suggests an increased risk for PHC development.

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