DO TYPES OF GROUND GLASS HEPATOCYTES THAT REPRESENT SPECIFIC HBsAg ENCODING GENE MUTATIONS CORRELATE WELL WITH STAGE OF FIBROSIS IN PATIENTS WITH CHRONIC HEPATITIS B VIRUS (HBV) INFECTION?

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ABSTRACT

Objective: *Specific subcellular localization of HBsAg protein on ground glass hepatocytes (GGH) were shown to represent the different mutations on HBsAg encoding gene.*

This study aims to investigate the importance of GGH types as a novel factor that may correlate with other clinicopathological variables, as well as an indicator for progression and severity of the disease in patients with chronic HBV (CHB) infection.

Material and Method: *Liver biopsies from 54 patients with CHB infection were evaluated. Specific topographic distribution of HBsAg protein on hepatocytes was visualized with immunohistochemical method. GGH's were typed into two groups based on immunomorphological expression patterns.*

Results: *There were 36 cases with GGH type 1, 14 with GGH type II and 4 cases with both types. Histological activity index(HAI) was minimal to absent (<1) in 12 cases (22%), was between 1-4 in 33 (61%) and was higher than 4 in 19 (35%) cases. High stage of fibrosis is strongly correlated with GGH type I ($p<0.005$). No significant correlation was observed between GGH types and other variables.*

Conclusion: *Different types of GGH correspond to specific pre-S mutations with deletions on pre S1 and S2 regions. In addition to be an histological sign of chronic HBV infection, the types of GGH may represent an another parameter that may contribute to progression or regression of fibrosis in patients with CHB.*

Key Words: *Ground glass heptocytes, HBsAg carrier, Pre S mutations, fibrosis.* ***Nobel Med 2011; 7(1): 23-28***

KRONİK HEPATİTİS B VİRUS (HBV) İNFEKSİYONU OLAN HASTALARDA, HBsAg GENİNDEKİ SPESİFİK MUTASYONLARI TEMSİL EDEN BUZLU CAM GÖRÜNÜMDEKİ HEPATOSİTLERİN TİPLERİ FİBROZİS İLE YÜKSEK KORELASYON GÖSTERMEKTE MİDİR?

Amaç: Karaciğer biyopsilerinde HBsAg taşıyıcılığı buzlu cam görünümündeki hepatositler olarak izlenir (BCH). HBsAg proteinin BCH içindeki subsellüler lokalizasyonun spesifik gen mutasyonlarına bağlı olduğu bildirilmiştir. Bu çalışmanın amacı bu gen mutasyonlarının yol açtığı morfolojik tipleri ile hastaların klinikopatolojik özelliklerini karşılaştırmaktır.

Materyal ve Metod: Kronik HBV infeksiyonu olan 54 hastanın karaciğer biyopsileri değerlendirildi. HBsAg proteinin hepatositler üzerindeki lokalizasyonu immünohistokimya ile incelendi. BCH tipleri daha önce bildirilen sınıflamaya göre yapıldı ve bu tipler klinik özellikler ile karşılaştırıldı.

Bulgular: Otuz altı olgu BCH tip 1, 14 olgu tip II ve 4 olgu mikst tip olarak değerlendirildi. Histolojik aktivite indeksi 12 olguda minimal (%22), 33 olguda 1-4 arasında (%61) ve 19 olguda (%35) ise 4'ten fazlaydı. Fibrozis derecesi tip 1 BCH ile yüksek korelasyon gösterdi ($p<0,005$). BCH tipleri ve diğer parametreler arasında önemli korelasyon saptanmadı.

Sonuç: BCH tipleri HBsAg genindeki pre S1 ve S2 bölgelerindeki spesifik mutasyonlar ile ilişkilidir. BCH tip 1 fibrozis ile yüksek korelasyon göstermiştir. BCH tipleri daha önce tanımlanmış klinikopatolojik parametrelere ek olarak hastaların monitorizasyonunda kullanılabilir. Ayrıca bu sonuçlar immün yanıttan kaçabilen bir HBsAg mutasyonun morfolojik olarak görülebildiğini göstermektedir.

Sonuç olarak karaciğer biyopsilerinde, HBS genindeki mutasyonları temsil eden BCH'lerin morfolojik tiplerinin değerlendirilmesinin bu hastalarda fibrozisin monitorizasyonu ve prognozunun belirlenmesinde yeni ufuklar açacağını düşünüyoruz.

Anahtar Kelimeler: Buzlu cam görünümünde hepatositler, HBsAg taşıyıcılığı, Pre S mutasyonları, fibrozis. **Nobel Med 2011; 7(1): 23-28**

INTRODUCTION

Hepatitis B virus (HBV) is a common infection and affects approximately two billion people worldwide.[1] The cellular injury and fibrosis that are the main mechanisms involved in chronic Hepatitis B virus infection (CHB) lead to long term complications of patients. Fibrosis and its related consequences such as cirrhosis and hepatocellular carcinoma are the main factors that burden the load on the patient care and are the main cause of HBV related deaths and long term complications.[2]

Histological sign of HBV infection in liver biopsies is the ground glass hepatocytes (GGH). Ultrastucturally the GGH is formed of abundant smooth endoplasmic reticulum (SER), which harbors the accumulation of Hepatitis B surface antigen protein (HBsAg). In histological sections, this abundant SER is observed as the hepatocytes with abundant granular cytoplasms, which resembles ground glass in light microscope.

There have been considerable advances in the understanding the mechanisms of hepatic fibrosis over last two decades. Authors now recognize that hepatic fibrosis is a dynamic process, which involves the host related and etiological agent related factors together.[2] Current study aims to investigate the relationships between the GGH types that represent specific mutations in HBsAg encoding gene and viral replication, clinical and histological, parameters, notably the fibrosis in patients with CHB.

MATERIAL and METHOD

We retrospectively evaluated the liver biopsy materials and case files of the patients with chronic HBV infection. In order to form a relatively homogeneous group, we included only the asymptomatic male patients that are 18-25 years old and previously undiagnosed. There were fifty four patients. All patients were young males. Patients did not receive prior antiviral treatment. None of them were intravenous drug abuser or HIV positive. All patients were seropositive for HBsAg, with detectable HBV DNA in their sera and seronegative for Hepatitis C virus. Of these 37 were HbeAG seropositive.

Liver biopsies were percutaneous trucut needle biopsies in all patients. All pathological material were processed in our laboratory with same standards. The biopsies were fixed in 10% neutralized formalin, routinely processed and parafin embedded. Four micron sections were cut and routinely stained with hematoxylin–eosin. In addition to hematoxylen and eosin stain, silver impregnation and trichrome stain were performed in each case. For immunohistochemical study, an additional 5 →

micron thick section was cut from paraffine block and taken on poly L lysine coated glass slide. Sections were deparaffinized and rehydrated through graded alcohols and rinsed in phosphate buffer saline solution. A prediluted biotinylated antibody for HBsAg (obtained from Dako, Türkiye) was applied to sections and incubated for 30 minutes in humid chamber. Hydroperoxide (0.3%) then was applied to inhibit endogenous peroxidase activity. Sections then incubated with labelled streptavidin biotine peroxidase (LSAB, obtained from Dako, Türkiye). Reaction products were obtained with 1% solution of 3,3 diaminobenzidine (DAB, Obtained from Dako, Türkiye) in tris buffer. Slides then were counterstained with Mayer's hematoxyline, dehydrated through graded alcohols, cleared in xylene and coverslipped. Positive reaction is identified as brown product on cytoplasms of GGH (Figure 1).

We reviewed all the histological material for confirmation of histological diagnosis and correct scoring according to modified Histologic Activity Index (HAI).[3] The stages of fibrosis were classified into no fibrosis, (stage 0), fibrosis confined to portal tracts (stage I), portal and periportal fibrosis with occasional porto-portal septa formation (stage II), bridging fibrosis (stage III), cirrhosis (stage IV).[4]

For this particular study we utilized the GGH typing proposed by Wang et al.[5] Type I GGH is defined as the presence of HBsAg on entire cytoplasm (Figure 1). This type of GGH's are scattered on hepatic nodules and observed as single cells (Figure 2). Type II GGH is characterized with an unique expression pattern. HBsAg is expressed in the peripheral parts of cytoplasm especially on the subsinusoidal pole of hepatocytes (Figure 3). This pattern also tend to cluster in nodules and lobules that lack of HBsAg expression are present (Figure 4).

Statistical analysis

The statistical analysis was carried out with SPSS version 11 (SPSS, Chicago, IL, USA). For the nonparametric tests, Mann Whitney U was utilized for data that were not normally distributed. The Spearman rank correlation test was used to investigate the correlations of GGH types and categorical variables. $P < 0.05$ was considered to be statistically significant.

RESULTS

The data of clinical and laboratory of the patients studied are given in Table 1. There are 12 patients with minimal to none histological activity index (HAI<1), 23 patients with HAI less than 4 and 19 patients with HAI >4. Breakdown of fibrosis is as follows: 34 patients with stage 0 (65%), twelve patients with stage I (22%), two patients with stage II (4%) and five patients with stage III (9%). There was no patient with cirrhosis (stage VI) in this study.

Immunohistochemically HBsAg expression were observed in all cases, however in 5 cases the expression was observed in less than 10% percent of all hepatocytes. Expression of HBsAg occured as diffuse cytoplasmic in 36 (67%), submembranous in 14 (26%) and mixed submembranous and diffuse →

Table 1: Clinical and laboratory data of cases

	HbeAg positive	HbeAg negative
Number of patients	36	18
Age (median-years)	21(19-23)	24 (18-25)
AST (U/l) (average)	43 (17-90)	54 (24-69)
ALT (U/l) (average)	47 (13-108)	122 (78-351)
HBV DNA IU/mL (median)	760000 (370-990000)	9000 (5000-800000)
Duration of HBsAg positivity until biopsy (median-months)	14 (7-24)	12 (6-84)

Numeric variables are given in median range. **AST:** aspartate aminotransferase, **ALT:** alanine aminotransferase

Table 2: Disease activity and HBsAg expression patterns.

	Carrier	%	Chronic hepatitis	%
Intrahepatocytic distribution of HBsAg				
Submembranous (GGH Type II)	3	27	11	26
Diffuse cytoplasmic (GGH Type I)	8	73	28	65
Mixed	0	0	4	9
Overall quantification of expression				
<10 %	1	9	4	9
10-50%	2	18	13	30
>50 %	8	73	26	60

Table 3: Stage of fibrosis and HBsAg expression

	Stage of fibrosis							
	0	%	I	%	II	%	III	%
Disease activity								
Carrier	11	30	2	17	0	0	0	0
Chronic hepatitis	24	65	10	83	2	100	5	100
Intrahepatocytic distrubution of HBsAg								
Submembranous (GGH Type II)	12	32	2	17	1	50	0	0
Diffuse cytoplasmic (GGH Type I)	23	62	6	50	1	50	5	100
Mixed	0	0	4	33	0	0	0	0
Overall quantification of expression								
<10 %	7	19	0	0	0	0	0	0
10-50%	4	11	6	50	0	0	3	60
>50 %	24	65	6	50	1	50	2	40

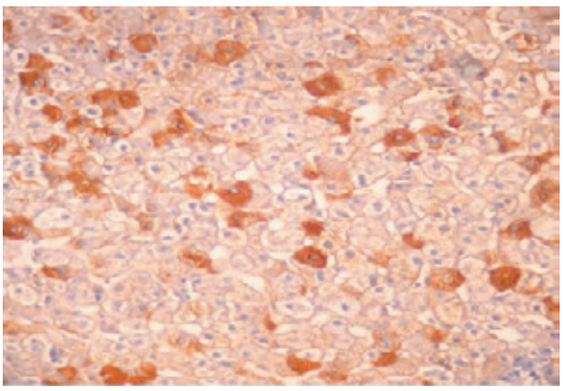

Figure 1. Type 1 GGH. Dense homogenous expression of HBsAg on entire Hepatocyte cytoplasm (HBsAg immunohistochemistry). Red to brown pigment denotes the HBsAg protein.

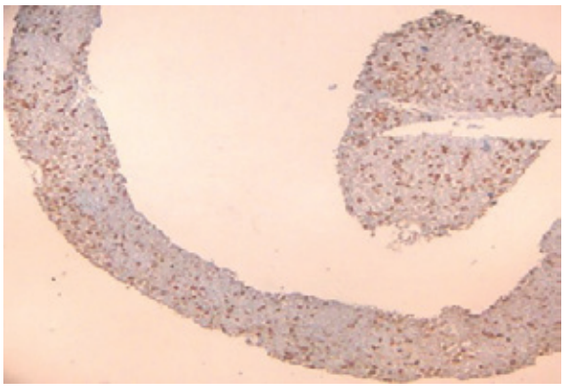

Figure 2. Scattered pattern of expression in GGH type 1. Note that HBsAg expression is seen on almost on lobules on biopsy material (HBsAg immunohistochemistry, scanning magnification)

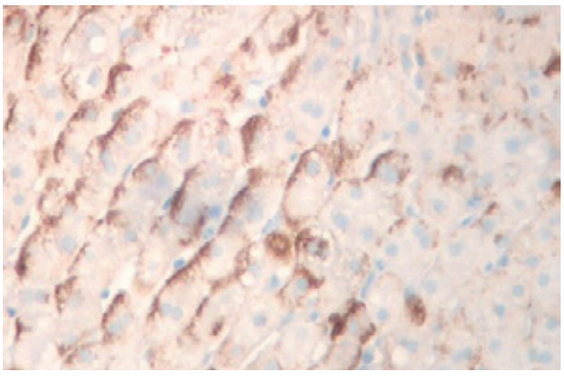

Figure 3. GGH type II. Subsinusoidal accumulation of HBsAg protein on hepatocytes. (HBsAg immunohistochemistry x400)

cytoplasmic in 4 (7%) cases. Breakdown of HBsAg expression patterns are given in Table 2. Stage of fibrosis and HBsAg expression pattern distribution are given in Table 3. There was a highly significant positive correlation between the GGH type II and low stages of fibrosis ($p<0.005$). No significant correlation between the types of GGH and other parameters such as histological activity index, HBV DNA, transaminase level and HBeAg positivity was found.

DISCUSSION

The GGH's that are recognized as histological hallmark of HBV carrier status , were shown to harbor the HBsAg protein in 1970's.[6,7] Since then several expression patterns of HBsAg on GGH were evaluated to correlate the histological and viral replication parameters.[8-11] Recently Wang et al defined two immunomorphological types of GGH that represent the specific pre S mutations in gene encoding the HBsAg protein.[5] Authors identified the type 1 GGH as diffuse cytoplasmic expressions on scattered hepatocytes. Type II GGH has been observed with a unique expression pattern in which HBsAg expression is limited to cell periphery. Type II GGH is also seen in hepatocytes that tend to cluster in nodules. These preferential accumulation of HBsAg protein on subcellular level also correlate with mutations on HBsAg encoding gene so that GGH type I corresponds to pre S1 deletion whereas GGH type II is associated with pre S2 deletion mutation.[5,12] Specific deletions on HBsAg encoding gene were shown to be interact with immune response of the host. HBV surface protein is encoded from three different segments on HBV gene.[13] The large surface protein is encoded from all three segments, middle surface protein from pre S1-pre S2 and small surface protein from the S region.[13,14] It was also shown that deletion at pre S2 sites associated with epitope of cytotoxic T cell and neutralization responses. This may represent an immun escape form and corresponds to the observation that GGH II clusters contain minimal or none inflammatory responses.[12,15,16]

Immunomorphological expression patterns of HBV antigens have been investigated in several studies previously.[9,17-22] Ramakrishna et al., correlated the nuclear and membranous staining patterns of HBV antigens with histological parameters.[18] Authors demonstrated that nuclear localization of HBcAg in HBeAg(-) group is significantly correlated with degree of fibrosis. However Kim et al did not find a significant correlation between HBcAg expression and degree of fibrosis.[19]

In addition to other HBV antigens, authors observed unusual patterns of HBsAg expression and investigated the correlation of these patterns with viral replication status. Parameters.[8-9-11,18,22] The results of different studies are not conclusive so far. Membranous pattern has been reported to associate with active HBV replication whereas cytoplasmic expressions were proposed to associate with histologically→

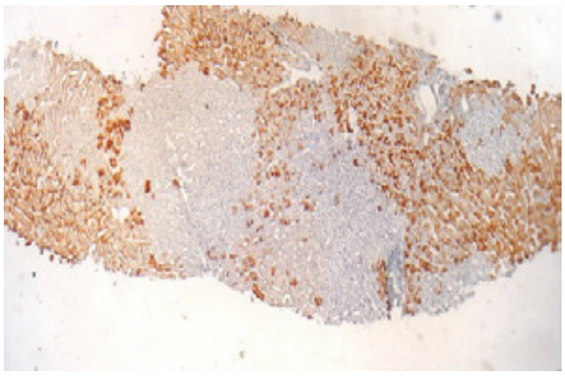

Figure 4. Distribution of type II ground glass hepaotcytes on liver. Notice the lobules that are free of HBsAg protein.

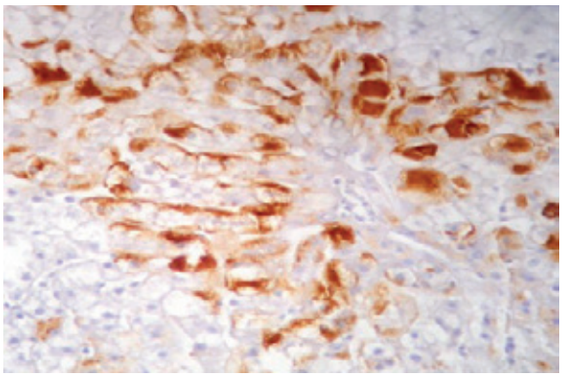

Figure 5. Simultaneous presence of GGH type I and II . (HBsAg immunohistochemistry X200).

inactive CHB.[10,23] However the results of the study by Wee et al contradicted with these reports.[9] Recently Ramaskishna et al showed that cytoplasmic expression of HBsAg correlated with the high viral replication.[18]

In current study GGH type 1 which corresponds to pre S1 mutation, was correlated with high degree of fibrosis. Previous studies showed that these type of HBsAg accumulation might be associated with slow viral replication.[8,10,22] These may seem paradoxical. In patients with low replication, surface gene integration may be present and a large quantitiy of HBsAg is probably produced as a part of transcription from hepatocyte gene.[9]This large quantity of HBsAg may play role in the formation of high level of fibrosis.

Our results showed that both types of GGH present together in four cases (Figure 5). This observation supports the previous proposal that GGH types may shift to another type during the seroconversion of HBeAg.[5,24] GGH types also shifts from GGH I dominance to GGH II dominance during the progression of CHB.[24] This morphological evidence also supports that the prevalence of pre S2 mutants gradually increases over the natural course of CHB in the serums and liver tissues of patients that progressed to hepatocellular carcinoma.

Hepatic fibrosis (HF) in chronic liver injury is dynamic and abundant evidence shows that HF, even the

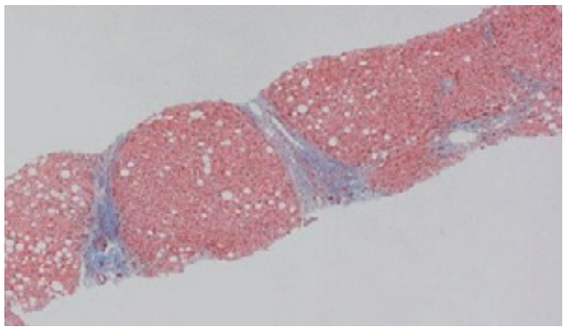

Figure 6. In trichrome staining, fibrosis is evident as blue colored bands in liver biopsy (trichrome stain X10).

cirrhosis is reversible.[25-28] The most important factor that reverses the HF is the elimination of etiological factor.[27-29] Eradication of HBV has been shown to reverse the HF even the cirrhosis in some patients.[26] HF is multifactorial process and involves the interactions inflammatory effector cells, fibroblasts, endothelial, bone marrow cells as well as chemical mediators (Figure 6).[28-32] We believe that in addition to internal host factors, deletions in etiological agent genes that result in different subcellular accumulation of HBV related antigens may contribute the dynamic process of HF.

We conclude that assessing GGH types and their associated mutant forms in liver biopsies, would implement novel aspects in monitoring and predicting the outcome of HF in patients with CHB.

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REFERENCES

1 Lee W. Hepatitis B virus infection. N Engl J Med 1997; 337: 1733–1745.

2 Rockey DC Current and future anti-fibrotic therapies for chronic liver disease. Clin Liver Dis 2008; 12: 939–962

3 Ishak K, Baptisa A, Bianchi L, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995; 22: 696–699.

4 Brunt E. Grading and staging the histopathological lesions of chronic hepatitis. The Knodell histological activity and beyond. Hepatology 2000; 31: 241-246.

5 Wang HC, Wu HC, Chen CF, et al. Different types of ground glass

hepatocytes in chronic hepatitis B virus infection contain specific pre-S mutants that may induce endoplasmic reticulum stress. Am J Pathol 2003; 163: 2441-2449.
6 Popper H. Ground glass hepatocytes as diagnostic hint. Hum Pathol 1975; 6: 517-520.
7. Hadziyannis S, Gerber MA, Vissoulis C, et al. Cytoplasmic hepatitis B antigen in 'ground-glass' hepatocytes of carriers. Arch Pathol 1973; 96: 327–330.
8 Hsu HC, Lai MY, Su IJ et al. Correlation of hepatocytic HBsAg expression with virus replication and liver pathology. Hepatology 1988; 8: 749–754.
9 Wee A, Yap I, Guan R. Hepatocyte hepatitis B surface antigen expression in chronic hepatitis B carriers in Singapore: correlation with viral replication and liver pathology. J Gastroentrol Hepatol 1991; 6: 466–470.
10 Chu CM, Liaw YF. Membrane staining for hepatitis B surface antigen on hepatocytes: a sensitive and specific marker of active viral replication in hepatitis B. J Clin Pathol 1995; 48: 470-473.
11 Mukhopadhya A, Ramakrishna B, Richard V, et al. Liver histology and immunohistochemical findings in asymptomatic Indians with incidental detection of hepatitis B virus infection. Indian J Gastroenterol 2006; 25: 128-131.
12 Su IJ, Wang HC, Wu HC, et al. Grond glass hepatocytes contain pre-S mutants and represent preneoplastic lesions in chronic hepatitis B virus infection. J Gastroentrol Hepatol 2008; 23: 1169-1174.
13 Fan YF, Lu CC, Chang YC, et al. Identification of a pre-S2 mutant in hepatocytes expressing a novel marginal pattern of surface antigen in advanced diseases of chronic hepatitis B virus infection. J Gastroenterol Hepatol 2000; 15: 519–528.
14 Chen BF, Liu CJ, Jow GM, et al. High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases. Gastroenterology 2006; 130: 1153-1168.
15 Tai PC, Banik D, Lin GI, et al. Novel and frequent mutations of hepatitis B virus coincide with a major histocompatibility complex class I-restricted T-cell epitope of the surface antigen. J Virol 1997; 71: 4852-4856.
16 Fan YF, Lu CC, Chen WC, et al. Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. Hepatology 2001; 33: 277-286.
17 Hsu HC, Wu TT, Wu MZ, et al. Evolution of expression of hepatitis B surface and core antigens (HBsAg, HBcAg) in resected primary and recurrent hepatocellular carcinoma in HBsAg carriers in Taiwan. Correlation with local host immune response. Cancer 1988; 62: 915-921.
18 Ramakishna B, Mukhopadhya A, Kurian G. Correlation of hepatocyte expression of hepatitis B viral antigens with histological activity and viral titer in chronic hepatitis B virus infection: An immunohistochemical study. J Gastroentrol Hepatol 2008; 23: 1734-1738.
19 Kim TH, Cho EY, Oh HJ, et al. The degrees of hepatocyte cytoplasmic expression of Hepatitis B core antigen correlate with histologic activity of liver disease in the young patients with chronic hepatitis B infection. J Korean Med Sci 2006; 21: 279-283.
20 Sharma RR, Dhiman RK, Chawla Y, et al. Immunohistochemistry for core and surface antigens in chronic hepatitis. Trop Gastroenterol 2002; 23: 16–19.
21 Dusheiko G, Paterson A. Hepatitis B core and surface antigen expression in HBeAg and HBV DNA positive chronic hepatitis B: correlation with clinical and histological parameters. Liver 1987; 7: 228-232.
22 Wu PC, Lau JY, Lau TK, Lau SK, Lai CL. Relationship between intrahepatic expression of hepatitis B viral antigens and histology in Chinese patients with chronic hepatitis B virus infection. Am J Clin Pathol 1993; 100: 648-653.
23 Serino Z, Varli M, Erden E, et al. Nuclear localization of hepatitis B core antigen and its relations to liver injury, hepatocyte proliferation and viral load. J Clin Gastroenterol 2003; 36: 269-272.
24 Su IJ, Lai MY, Hsu HC, et al. Diverse virological, histopathological and prognostic implications of seroconversion from hepatitis B e antigen to anti-HBe in chronic hepatitis B virus infection. J Hepatol 1986; 3: 182-189.
25 Friedman SL, Rockey DC, Bissell DM. Hepatic fibrosis 2006: report of the Third AASLD Single Topic Conference. Hepatology 2007; 45: 242-249.
26 Mallet VO, Dhalluin-Venier V, Verkarre V, et al. Reversibility of cirrhosis in HIV/HBV coinfection. Antivir Ther 2007; 12: 279-283.
27 Abergel A, Darcha C, Chevallier M, et al. Histological response in patients treated by interferon plus ribavirin for hepatitis C virus-related severe fibrosis.Eur J Gastroenterol Hepatol 2004; 16: 1219-1227.
28 Russo FP, Alison MR, Bigger BW, et al. The bone marrow functionally contributes to liver fibrosis. Gastroenterology 2006; 130: 1807-1821.
29 Lai CL, Chien RN, Leung NW, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998; 339: 61-68.
30 Bahcecioglu IH, Koca SS, Poyrazoglu OK, et al. Hepatoprotective effect of Infliximab, an anti-TNF-alpha agent, on carbon tetrachloride-induced hepatic fibrosis. Inflammation 2008; 31: 215-221.
31 Rockey DC, Chung JJ. Endothelin antagonism in experimental hepatic fibrosis. Implications for endothelin in the pathogenesis of wound healing. J Clin Invest 1996; 98: 1381-1388.
32 Kinnman N, Francoz C, Barbu V, et al. The myofibroblastic conversion of peribiliary fibrogenic cells distinct from hepatic stellate cells is stimulated by platelet derived growth factor during liver fibrogenesis. Lab Invest 2003; 83: 163-173.