

Significance of apoptotic markers Bcl-2 , CD4+ T cells , hepatocyte growth factor and metalloproteinase -9 in infected patients with HCV

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ABSTRACT

To examine the possible involvement of human B-cell leukemia /lymphoma 2(Bcl2) , CD 4+ cells , hepatocyte growth factor (HGF) and metalloproteinase -9 (MMP-9), in the development of liver diseases caused by HCV infection, serum activities of these biomarkers were demonstrated by quantitative determination of Enzyme Linked Immunosorbant Assay(ELISA) . Two groups of subjects (60 for each) were examined in this study: healthy control and patients with chronic hepatitis C(HCV) The results showed significant decrease in Bcl-2 ($P \leq 0.0001$) , and CD 4+ ($P \leq 0.001$) , while significant increase in HGF and MMP-9($P \leq 0.05$). These findings imply an influence of these biomarkers by the existence of virus that might influence the following progression of liver disease, and a distinction between the pathological mechanisms of HCV. Since the serum MMP-9 activity were significantly varied between each stage of liver disease, an individual profile of these parameters might serve as an easy accessing serum marker to monitor the progression of liver disease.

INTRODUCTION

Hepatitis C virus (HCV) causes a prolonged and persistent infection that progresses to liver cirrhosis and eventually induces an approximately 400-fold increase in the risk of developing hepatocellular carcinoma. The mechanisms responsible for viral persistence and disease progression in HCV infection are not well defined; after infection is established, multiple factors influence the host-virus interaction resulting in a unique individual disease pattern. Apoptosis is a physiological form of cell death evolved in multicellular organisms to eliminate unwanted cells through a coordinated series of enzymatic steps and controlled by inhibitors at each step. The Bcl-2 proto-oncogene is an established survival factor whose physiological function is to prevent apoptosis, and the activity of Bcl-2 may be counteracted by dimerization with bax. Bax is proapoptotic and, together with other death agonist members, increases sensitivity to death-inducing signals.

It has been reported that some viral infections influence the susceptibility of peripheral blood mononuclear cells (PBMC) to apoptosis, and this may be a plausible mechanism describing the insufficient antiviral immune response leading to persistent viral infection and disease progression (Hanafy et al ., 2010). One hundred and seventy million people (~3% of the world's population) are chronically infected with hepatitis C virus (HCV) (Houghton , 1996). Viral persistence is seen in the majority of the infected people leading to cirrhosis, liver failure and cancer. Treatment with pegylated IFN α and ribavirin shows only ~50% success rate and is also dependent upon the genotype of HCV involved(Wolf and Green, 1999). However, a strong cellular immune response mediated by CD4+ and CD8+ T cells, has been shown to clear the virus after an acute infection (Feuillard et al., 2000). Therefore, amending the immune system to mount vigorous antigen-specific T cell responses could be an attractive immunotherapeutic approach to combat HCV infection.

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Studies in HCV-infected patients suggest that the presence of regulatory T cells, immunosuppressive cytokines and inhibitory receptors might contribute to HCV persistence (Silverman *et al.*, 1994). On the other hand, a multi-specific, strong and sustained T cell response is correlated to HCV viral clearance (Sedar, 2005). Expansion of antigen-specific CD4+ and CD8+ T cells are particularly important in controlling viral replication. These cells provide viral clearance by producing antiviral cytokines and by mediating their direct cytolytic mechanisms. Bowen and Walker (2005) evidently showed the role of both CD4+ and CD8+ T cells in HCV viral clearance. Strong and sustained CD4+ and CD8+ T cell responses together lead to the resolution of HCV. Also, DCs play a major role by processing and presenting the antigens on MHC-I and MHC-II molecules and providing optimum stimulation to CD8+ and CD4+ T cells, respectively. (Krishnadas *et al.*, 2010).

Virus-specific CD4+ T cells play a major role in hepatitis C virus (HCV) infection. While spontaneous resolution is associated with vigorous and multispecific CD4+ T cell responses, chronic infection is associated with weak or absent HCV-specific T cell responses (Mueller *et al.*, 2010). Elimination of hepatitis C virus (HCV) has been repeatedly linked to the generation and maintenance of strong multi-epitopic CD8+ cytolytic T lymphocyte (CTL) responses (Day *et al.*, 2002). However, CD8+ CTL alone do not seem to be sufficient for HCV clearance. Efficient control of HCV infection by virus-specific CTL appears to be critically dependent on help provided by the activation and maintenance of strong CD4+ type 1 T helper lymphocyte (TH1) responses (Gerlach *et al.*, 1999). In chronic hepatitis C, however, both virus-specific CD4+ TH1 and CD8+ CTL show sustained dysfunction *in vitro* (Langhans *et al.*, 2006). Concerning with HGF, is produced in various organs of the body and is characterized as a multifunctional protein with various biological activities. Interestingly, although increased HGF levels in serum or tissue have been reported in patients with HCC and HCV, there was no significant difference of the HGF levels in patients with HCC, non-HCC tumor and chronic hepatitis group. However, patients in these three groups did have a higher HGF level compared to normal subjects. It is well known that HCC is frequently associated with chronic hepatitis B or C infection and liver cirrhosis. A major function of a tumor marker is that the marker should be able to differentiate patients who truly have cancer from those who do not. HGF levels may also be elevated in liver disease-associated conditions other than HCC and HCV, suggesting measurement of HGF level is not helpful in differentiating HCV from other non-HCC conditions. The previous study failed to support a direct relationship of HGF and human HCV and argued against the role of HGF in HCV. (Hsia *et al.*, 2007).

As for HCV, as many as 20% of chronic hepatitis C patients go on to develop cirrhosis in the first decade or two of HCV infection (Yano *et al.*, 1996). It has been estimated that as many as 1 to 4% per year of patients with established cirrhosis may go on to develop hepatocellular carcinoma (Di Bisceglie,

1997). A major pathohistological change during the progression of liver diseases (from hepatitis to cirrhosis, then to hepatocellular carcinoma) is the quantity of extracellular matrix proteins (ECM), that is excessively accumulated in the fibrotic or cirrhotic liver and is dissolved in the hepatocellular carcinoma to facilitate cancer invasion. Degradation of the extracellular matrix is associated with most physiological and pathological processes requiring tissue remodeling by the matrix metalloproteinase (MMP) superfamily.

The deregulation of the activity of MMPs is thought to be one of the factors responsible for the pathological alteration of the ECM in liver diseases. Several studies have examined the activities of MMPs and their inhibitors in the patients with liver diseases (chronic hepatitis, liver cirrhosis and hepatoma), from the view point of studying their role in the development of the diseases and evaluating their possible application in serving as a serum marker (Kuo *et al.*, 2000). Thus, the present study aims to use the Bcl₂ CD4 cells, HGF and MMP-9 to demonstrate their role in the development of HCV disease and evaluating their possible application in serving as a serum marker.

METHODS AND SUBJECTS

Methods

The informed consents were taken from the parents of our studied groups according to guideline of the Medical Ethical Committee of National Research Centre, Dokki, Giza. All the studied groups were subjected to full history report including personal history, complete present history, family history, social history and past history.

Clinical examination and subjects

This study included one group of healthy control subjects and one group of patients diagnosed with chronic hepatitis C and were obtained from National Institute of Endemic Diseases Research and Liver, Cairo, Egypt. Each group consisted of 60 subjects. Blood was drawn by vein puncture into Venoject tubes in the morning after an overnight fast. Blood was centrifuged at 3000 rpm and serum was used for all the laboratory determinations including the apoptotic markers (Bcl-2), CD4+ T cells, hepatocyte growth factor (HGF), and the activity of metalloproteinase -9 (MMP-9). For healthy controls, blood samples were drawn during a medical check up and were normal for blood biochemical and immunological examinations.

Determination of serum apoptotic marker human B-cell leukemia /lymphoma 2(Bcl2)

The serum activity of human B-cell leukemia /lymphoma 2(Bcl2) was measured by Enzyme linked Immunosorbent Assay (Elisa) according to the assay method of Bauer and Bryant (2004).

Determination of serum CD4

The serum activity of human CD4+ was measured by human CD4 Elisa kits (Munn *et al.*, 2004).

Determination of serum HGF

Human hepatocyt growth factor (HGF), was measured by Elisa kit according to the methods determined by Yoshinaga et al.(1993)

Determination of serum matrix metalloproteinase-9(MMP-9)

The serum activity of human MMP-9 was measured by Elisa kit according to Borkakoti (1998)

Statistical analysis

The data were presented as means \pm SD of 60 subjects . The statistical significance of the means was determined by T-test (SPSS), statistical computer programme , where $p \leq 0.05$ (*), $p \leq 0.001$ (**) and $p \leq 0.0001$ (***) .

RESULTS

The present results (as indicated in Table 1 and Fig. 1), show that Bcl2 significantly decreased ($P \leq 0.0001$) in serum of HCV as compared to normal healthy patients with percentage decrease amounting to 74.54 %. In addition, significant decrease in CD4+ ($p \leq 0.001$), cells in patients of HCV with percentage decrease reached to 17.04 % ,was observed comparing to normal patients . Although, significant increase in serum HGF and MMP-9 ($P \leq 0.05$) , was detected in serum of HCV with percentage increase of 65.67 and 31.17 % , respectively as compared to normal controls.

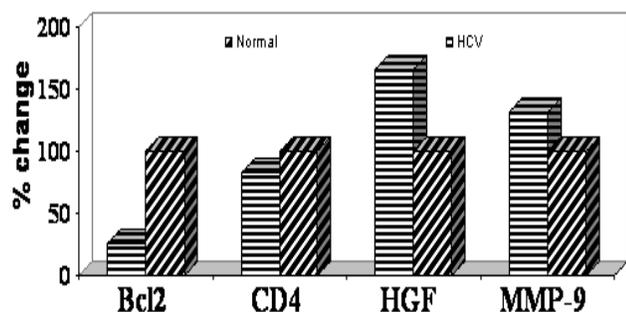


Fig. 1 : Percentage change in Bcl2, CD4, HGF and MMP-9 in HCV and normal healthy control patients.

Table. 1 : Levels of Bcl2-, CD4+, HGF and MMP-9 in controls and HCV subjects.

Parameters	Groups	Mean \pm Deviation
BCL2	cont	3.7240 \pm .5160
	hcv	2.9766 \pm .2768***
CD4	cont	4.0030 \pm 1.6472
	hcv	3.3189 \pm 1.0785**
HGF	Cont	4.2693 \pm .8550
	Hev	7.06 \pm 9 \pm 0.54***
MMP-9	cont	259.6200 \pm 36.3237
	hcv	340.3264 \pm 35.4702

DISCUSSION

Hepatitis C virus (HCV) is often established as a persistent infection to cause chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma which is a significant health problem around the world. Despite advances in the knowledge of the

molecular virology of hepatitis C virus (HCV), the mechanisms of hepatocellular injury in HCV infection are not completely understood. After infection is established, multiple factors influence the host-virus interaction, resulting in a unique individual disease pattern. The available reports are in favor of a destructive mechanism mediated by the host immune system rather than a direct cytopathic effect of the virus (Nelson, 2001). This comprises both a non specific immune response and HCV specific humoral and cellular immune response (Nelson, 2001). A delicate balance normally exists in the body between the antiapoptotic and proapoptotic regulators of apoptosis to ensure the proper survival and turnover of different body cells (Wolf and Green, 1999). Imbalance in the apoptotic pathway occurs in disease scenarios (Feuillard et al., 2000).

The present study reveal, significant decrease in Bcl2 in serum of HCV patients. Previous studies demonstrate that apoptosis has been included in the cytopathic effect of HCV infection (Iken et al., 2006). Deng et al. (2008) suggested that HCV induced apoptotic cell death induced by Bax, a proapoptotic member of the Bcl-2 family, which is activated by HCV infection and induced apoptosis of the host cell through a Bax-triggered mitochondria mediated, mechanism. Also, it has been suggested that the Bcl-2 (antiapoptotic) to Bax ratio determines the cell susceptibility to apoptosis (Hanafy et al., 2010). It was found that , CD4+ ,CD8+ T lymphocyte and CD4+ monocyte subsets to apoptosis and elevated , under the apoptotic stimulus in patients with advanced chronic hepatitis compared with healthy control, and that the susceptibility of all peripheral blood mononuclear cell (PBMC) subsets to apoptosis had been increased with the escalating severity of the liver disease. In a good agreement with the present study , Hanafy et al .(2010), found , intracellular expression of Bcl-2 that is statistically significantly decreased in patients with advanced chronic HCV hepatitis. This suggested a molecular, mechanism in which the PBMC subsets became susceptible to apoptosis presumably because of the down-regulation of Bcl-2 expression during persistent viral infection(Hanafy et al ., 2010).

In addition, the significant down regulation of Bcl2 percentage expression in peripheral blood lymphocytes together with a statistically significant increased expression of Bax and a significantly decreased Bcl 2/Bax ratio were observed in patients with chronic HCV infection. This dysregulation of the apoptotic process could explain the progression of HCV infection in this patient group.

The present data show also, significant decrease in CD4 in serum of HCV patients it was found that eradication of viral infections probably depends on classical T helper (CD4+) and cytotoxic (CD8+) T-lymphocyte responses. Gerlach et al. (1999) found that in patients with acute HCV infection if the T-helper (CD4+) immune response was weak, less efficient or not maintained for a sufficient length of time, patients would proceed to persistent infection and chronic hepatitis. Furthermore , Hoffman et al. (1995), found that HCV-RNA positive individuals without clinical or histopathologic evidences of liver disease had a

statistically significantly higher CD4+ proliferative response to the HCV core protein than patients with chronic hepatitis, suggesting a protective role of CD4+ cells against hepatocellular damage .

One of the main contributors to the HCV genome is the hypervariable region 1 (HVR1), It has been shown that acute resolving hepatitis is associated with an evolutionary stasis of the HVR1 quasispecies, whereas progressive hepatitis correlates with sequence variability (Farci *et al.*, 2000). HVR1 is described as the main target of anti-HCV neutralizing antibodies and HVR1-specific CD4+ and CD8+ T cells have been correlated with the benign course of HCV infection (Tsai *et al.*, 1998). Moreover, the emergence of HVR1 variants within HVR-1-specific B and T cell epitopes with antagonistic functions suggested that this region may also concur to limit cellular immune response and favor viral escape (Tsai *et al.*, 1998). It has been suggested that HVR1 variations resulted from the action of a continuous immune-driven positive selection ,probably controlled by humoral immune responses(Scotta *et al.*, 2008).

Available evidence suggested that HVR1 variation is related to adaption and results from a continuous selection process, probably controlled by humoral immune responses (Scotta *et al.*, 2008). While, HVR1-specific CD4+ T cells can be evocated exclusively in resolving patients, all patients present anti-HVR1 antibodies. This suggested that, despite the co-expression on HVR1 of B and T epitopes, the presence of anti-HVR1 antibodies is independent of that of HVR1-specific T cells. Moreover, anti-HVR1 antibodies are characterized by IgG1 isotype, suggesting that, the levels of antigen below the threshold necessary to induce and maintain an efficient humoral immuneresponse could fail to mediate IgG isotype switch . Another finding is the difference between the cross-reactive nature of B and T cell epitopes. T cell epitopes are cross-reactive with unrelated HVR1 sequences, while B cell epitopes are not. The lack of HVR1-specific CD4+ T cells in patients evolving to chronic infection could be due to modifications of HVR1 sequences responsible for defective immunogenicity. No evidence that resolvers recognize epitopes different from those isolated from patients where the virus persists supporting the conserved nature of HVR1-B and T cell epitopes (Penin *et al.*, 2001). Thus, HVR1-specific CD4+ T cell response can be correlated with recovery from HCV infection, while anti-HVR1 antibodies are independent of the outcome of infection since their appearance in resolving patients occurs after viral clearance. Therefore they influence HVR1 variability only in patients with chronic evolution. Consequently, HVR1 complexity could represent a virus adaptive strategy to escape the continuous selective process mediated by anti-HVR1 antibodies (Scotta *et al.*, 2008).

Concerning HGF, the present results indicate ,significant increase in serum of HCV patients . HGF is a pleiotropic substance with mitogenic, morphogenic, motogenic and tumor-suppressor activity. HGF and its receptor are the key factors in liver regeneration (El-Serag and Mason,1999). HGF administration reduced hepatotoxicity and increased survival in various animal models of liver damage(Chang *et al.*, 2004). HGF also has

antifibrotic properties: in a rat model of dimethylnitrosamine induced fibrosis, HGF infusion improved liver histology and collagen content. Thus ,hepatocyte growth factor (HGF) is a potent stimulator of hepatocyte growth and an important regulator of liver regeneration in response to injury. In the liver, HGF is secreted from non-parenchymal cells (mainly Kupffer cells and sinusoidal endothelial cells). Normal human hepatocytes do not themselves produce HGF. Serum HGF is elevated in various liver diseases including acute and chronic hepatitis, liver cirrhosis, and HCC. Hepatocyte growth factor is a multifunctional cytokine and has been implicated in the pathogenesis of several tumors. Hepatocyte growth factor increases the motility and invasiveness of some cancer cells, both under *in vitro* and *in vivo* conditions. Recent studies have shown that HGF stimulates the invasiveness of human HCC cells, (Monvoisin *et al.*, 1999 , Wu *et al.*, 2006 and Chau *et al.*, 2008), and that high serum HGF levels in HCC patients are associated with tumor metastasis. Elevated HGF levels may be detrimental to the postresectional survival of HCC patients. The effects of HGF are mediated by a specific receptor encoded by the c-met protooncogene. The rise in portal HGF concentration in HCC patients undergoing hepatic resection may be caused by hepatocellular dysfunction and necrosis, with release of HGF by activated hepatic macrophages (Junbo *et al.*, 1999). Ljubimova *et al.* (1997), revealed specific HGF staining in HCC cells, supporting the existence of both autocrine and paracrine mechanisms of HGF action in HCC. Yamagami *et al.*(2002), indicated that in HCC tissue, HGF was observed in infiltrating mesenchymal cells and in the cytoplasm of cancer cells. It is reasonable that in the early phase after tumor resection, it is the surgery and part of the normal early healing process rather than the tumor cells are associated with postoperative increases in HGF concentrations. It has been suggested that there are continuous stimuli for hepatocyte regeneration *via* HGF/c-met interaction in chronic liver disease tissues. The regulatory and biological mechanisms of serum HGF and HCC recurrence may be complex. Nagata *et al.*(2001), reported that an HGF activator and HGF activator inhibitor exist in human HCC and act in concert to regulate HGF activity in the peri cellular microenvironment. Hepatocyte growth factor therapy has been studied for treating chronic hepatitis and liver cirrhosis .

Furthermore, In various liver diseases, hepatic HGF is located in non-parenchymal cells such as hepatic stellate cells, and also in endothelial and Kupffer cells (Tomiya *et al.*, 1998). There is also some evidence that polymorpho nuclear neutrophil (PMN) could be a source of HGF. Wolf *et al.* (1991), reported immunocytochemical data showing the presence of HGF in human bone-marrow PMN . Likewise, Sakagushi (1994) found HGF-immunostained PMN in the sinusoids of hepatic lobules and in the portal area of diseased liver . There is increasing evidence that PMN play a role in the pathophysiology of alcoholic hepatitis (AH). Indeed, neutrophilia is often frequent, and the liver is infiltrated by PMN. We recently showed that blood PMN are hyperactivated during AH, as they produce high amounts of reactive oxygen species (ROS), proinflammatory cytokines and

chemokines maintaining liver inflammation (Taieb *et al.*, 2000), PMN infiltration is also a factor of good prognosis, as corticosteroid therapy, the reference treatment of severe acute alcoholic hepatitis (AH) is particularly effective in patients with marked neutrophilia or liver PMN infiltration. Singla *et al.* (2010), postulated that activated PMN were a source of HGF in AH, and could therefore participate in tissue repair. Blood PMN from cirrhotic patients with and without AH were isolated and compared for their capacity to secrete HGF. Although quite a few investigators have discussed the roles of MMPs in liver disease, most of them had focused on their participation in cancer development and metastasis, some in liver cirrhosis, and few in chronic hepatitis. No study has been attempted to assess their role in the carriers of hepatitis viruses, and relate their activities to the possible progression of liver diseases. The present results show significant increase in the MMP-9 in the subjects that were positive for anti-HCV indicating influenced on the serum type IV collagenases activities by the existence of a virus. It was found that, the presence of HBeAg, a marker of active viral replication (Lee, 1998), in circulation is accompanied by the highest activity of MMP-2 and an inversely correlated, lowest activity and/or significant change of MMP-9 among all populations and *visa versa* (Capone *et al.*, 2012). This observation suggested that, the replication of HBV might cause increased secretion of MMP-2 that might, in turn, enhanced the inflammation of liver tissue and lead to chronic hepatitis, since an elevated activity of MMP-2 has been related to inflammation in several systems. When the patients presented chronic hepatitis B, the serum MMP-2 was at a mean activity that was significantly lower than that of healthy controls, while MMP-9 was significantly elevated (Kumagai *et al.*, 1999). Although previous studies had stated controversial results concerning the serum activity of MMP-2 in chronic liver diseases, they were concentrated on the chronic hepatitis C or non-discriminate chronic hepatitis patients (Walsh *et al.*, 1999). The activity of this parameter in chronic hepatitis B patients had not been documented previously. The papers of Walsh *et al.* (1999) and Ebata *et al.* (1997), reported elevated serum MMP-2 activities in 43 chronic hepatitis C patients and in 264 patients with chronic hepatitis (including 192 with chronic hepatitis C) respectively. Another study by Murawaski *et al.* (1999), found no significant difference in serum MMP-2 activities between chronic hepatitis C group and control group, while significant increase in MMP-9 level in serum of HCV patients. The subjects positive for HCV antibody, HBsAg, or both HBsAg and HbeAg, and the patients with different forms of chronic hepatitis also displayed significant, but less profound, differences in the serum MMP-9 activities. The MMP-9 activities of HCV and HBV showing, a distinguished variance in the MMP-2 and MMP-9 activities among the virus carriers and chronic hepatitis patients suggested that individual response in these parameters on the viral infection might be an indicator of pathogenic difference between HCV and HBV (Murawaski *et al.*, 1999). As chronic liver disease progresses to fibrosis, there is a pathological accumulation of extracellular matrix proteins whose destruction is a requisite initial step for the

following tumor metastasis and invasion. The imbalance between the metalloproteinases and their inhibitors is considered to be the major reason for the excess deposition and degradation of extracellular matrix proteins during the progression of liver diseases. In consistent with our present data, most studies found an elevated activity of MMP-9 in the serum or tissue of liver hepatoma patients of HCV (Arii *et al.*, 1996). Although the high activities of MMPs give a good explanation for the decomposition of extracellular matrix in hepatoma tissue, they are in conflict with the fibrotic histology in cirrhosis tissue. We postulated that the pathological significance of a disease stage is the result of the MMP activity of a previous stage. Using HBV infection, for example, the significantly above-normal activity of MMP-2 in HBV carriers might enhance liver inflammation and cause the occurrence of chronic hepatitis. On other hand, the extremely low activity of MMP-2 in the chronic B patients could help the progression into cirrhosis, at which stage MMP activity increases and might lead to cancerous pathology. A similar, but less significant, pattern in the changes of MMP-2 activities relative to the stages of disease was also observed in HCV patients (Ebata *et al.*, 1997). However, further studies connecting the activities of MMPs to the degree of liver fibrosis or inflammation are required to resolve the postulation. Moreover, the inhibitors of metalloproteinase 1 and 2 (TIMP-1 and TIMP-2) were found to be increased in the liver cirrhosis and hepatoma patients (Ebata *et al.*, 1997). This further complicates the pathological significance of the metalloproteinase system in the development of liver disease. Active replication of HCV indicated by the existence of HCeAg in the circulation resulted in notable changes in the activities of these two enzymes. These findings suggested that low replication activity of viruses already had an influence on the balance of the MMPs system that might affect the following progression of liver disease. The significant differences in the activities of MMP-2 and MMP-9 between the chronic hepatitis C and chronic hepatitis B patient also implied a distinction between the pathological mechanisms of HCV and HBV. Since the serum MMPs activities were significantly different between each stage of liver disease, an individual profile of these parameters might serve as an easy accessing serum marker to monitor the progression of liver disease. Chronic hepatitis C patients were significantly higher than the activities of healthy control. Our observation is in a line with the previous report that studied the plasma MMP-9 activity in liver diseases showing elevated MMP-9 activities in hepatocellular carcinoma, as other groups (chronic hepatitis and liver cirrhosis) comparable to control activity (Hayasaka's *et al.*, 1996). However, the below-control activity of MMP-9 in HCV might therefore emphasize the importance of MMP-2. Although the various forms of chronic viral hepatitis are similar in the clinical symptoms, biochemical abnormalities and histologic characteristics and in the ability to cause cirrhosis, the two viruses (HCV and HBV) are quite distinct (Kuo *et al.*, 2000). Thus, it could be concluded that Bcl₂, CD4+ cells, HGF and MMP-9 might be applied in serving as a serum biomarkers to demonstrate the development of HCV patients.

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