THE EGYPTIAN JOURNAL OF IMMUNOLOGY Vol. 17 (2), 2010
Page: 81-90

Genetic Profiling of Non-Hodgkin's Lymphoma with or without Hepatitis C Virus Infection

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Hepatitis C virus (HCV) which is one of the endemic viral infections in Egypt is not only hepatotropic, but also a lymphotropic virus and has many extrahepatic manifestations as mixed cryoglobulinemia and non-Hodgkin's lymphoma. We studied gene expression profile of 20 B-cell non-Hodgkin's lymphoma with HCV infection and 20 B-cell non-Hodgkin's lymphoma without HCV infection as a control group by c-DNA microarray. Out of the 15,500 studied genes, more than 1000 genes were differentially expressed; either upregulated or downregulated. We found that HCV may rescue B lymphocytes from apoptosis possibly through causing suppression of CASP1 and CASP4 and causing overexpression of the anti-apoptotic BCL2 gene. Also, HCV was associated with overexpression of the genes related to myeloid/lymphoid leukemia and B lymphoma as MLLT3, BAL, influences the overexpression of transcription regulator genes as TATA box binding protein (TBP) and may influence the overexpression of some immunoglobulin genes as immunoglobulin superfamily containing leucine gene in B cells resulting in overproduction of immunoglobulins in B-lymphocyte disorders. Moreover HCV was associated with reduced expression of MHC class II molecules in B lymphocytes, and therefore inhibition of antigen processing and presentation through downregulation of different MHC class II molecules genes. We conclude that the upregulated and the downregulated genes identified through the studied expression profiles of NHL with HCV infection may shed light on the mechanisms of HCV lymphomagenesis.

Hepatitis C virus (HCV) is recognized as a major threat to global public health. An estimated 170 million people worldwide are infected, most of them chronically infected and at risk for liver cirrhosis and hepatocellular carcinoma (HCC) (Alter et al., 1999). Egypt has possibly the highest HCV prevalence in the world where 10-20% of the general population is infected. HCV is the leading cause of HCC and chronic liver disease in the country (Habib et al., 2001). Approximately 90% of Egyptian HCV isolates belong to a single subtype, 4a, which responds less successfully to interferon therapy than other subtypes (Miller & Abu-Raddad, 2010).

Epidemiological studies suggest that HCV infection may play a direct role in the genesis of B-cell lymphoproliferative disorders, and clonal B lymphocytes are frequently detected in the blood and liver of patients with chronic HCV infection. Furthermore, antiviral treatment for HCV is associated with the regression of B-cell lymphoma. Nevertheless, how HCV induces B-cell lymphoproliferative disorders and whether HCV plays any role in B-cell immunity is still unclear (Gisbert *et al.,* 2003). The molecular basis for viral induced B cell proliferation is still unknown; one possibility is that HCV stimulates the proliferation of monoclonal B cells via their HCV-specific B cell receptor (BCR) on the cell surface. Binding of the HCV envelope proteins to a cellular ligand, CD81, may also enhance this antigen-driven process (Wunschmann *et al.,* 2000; Rosa *et al.,* 2005). Lymphomagenesis is a multifactorial process in which genetic, environmental and infectious factors can be involved (Fornasieri *et al.,* 2000).

The aim of this work was to study the molecular basis for HCV induced B cell proliferation and non-Hodgkin's lymphoma and to assess the lymphomagenetic properties of hepatitis C virus.

Patients and Methods

Patients

The study included 40 patients who attended the National Cancer Institute (NCI), Cairo University, and were consecutively diagnosed as B-cell non-Hodgkin's lymphoma. Tumors and their adjacent non-neoplastic tissues together with venous blood samples were obtained from patients. Tissues were immediately cut into three parts; one piece was processed for routine histolopathological examination to confirm diagnosis, determine the pathological features of the tumor and assess tumor: normal ratio. The second and third portions were immediately snap-frozen and stored in liquid nitrogen for RNA and DNA extraction.

Gene expression profiling was conducted with RNA samples from lymph node biopsies of two groups. From 20 B-cell NHL patients with HCV infection and 20 B-cell NHL patients without HCV infection as a control group. Gene expression profiling was conducted using microarray chips (Fox Chase Cancer Center) containing about 15,500 human probes, more than 1000 genes were differentially expressed. Among them, significantly expressed genes, either upregulated or downregulated, were compared based on greater than 2-fold ratio changes with significant P value <0.05. These include selected clusters of genes related to apoptosis, myeloid/lymphoid leukemia and B lymphoma, transcription regulation, immunoglobulin and MHC class II molecules.

Serological Markers

Antibodies to HCV were detected with HCV EIA version 3.0 (Innogenetics, Belgium). All serologic assays were done according to manufacturer's instructions.

Detection of HCV-RNA

RNA was extracted from patients' sera according to manufacturer's instructions by Qiagen (Qiagen, GmbH, Germany). The RT-PCR was performed as previously described by Zekri et al. (2002).

cDNA Microarrays

RNA extraction from tissues: RNA was prepared from tumor samples and their adjacent non-neoplastic tissues. Each sample was tested in triplicate on array 15K (Array-I) supplied from Fox Chase Cancer Center.

Briefly, RNA was extracted by homogenization (Polytron; Kinematica, Lucerne, Switzerland) in TRIzol reagent (Gibco BRL) at maximum speed for 90–120s. The homogenate was incubated for 5 min at room temperature. A 1:5 volume of chloroform was added, and the tube was vortexed and subjected to centrifugation at 12,000 g for 15 min. The aqueous phase was isolated, and one-half of the volume of isopropanol was added to precipitate the RNA. Purification was then performed with the Qiagen RNeasy Total RNA isolation kit according to manufacturer's specifications (Qiagen, Germany). The purified total RNA was finally eluted in 10 ul of diethyl pyrocarbonate-treated H2O, and the quantity and integrity were characterized using a UV spectrophotometer (Nanodrop). RNA was electrophoresed on an ethidium bromide stained agarose gel. It showed discrete bands of high molecular weight RNA between 7 Kb and 15 Kb, two predominant ribosomal RNA bands at approximately 5 Kb (28S) 2 Kb (18S), and low molecular weight RNA between 0.1 and 0.3 Kb (tRNA, 5S). The isolated RNA has an A 260/280 ratio of 1.9–2.1.

RNA Labeling

Probes for microarray analysis were prepared from RNA templates by the synthesis of first strand cDNA containing amino-allyl-labeled nucleotides (Sigma Cat # A0410), followed by a covalent coupling labeled cDNA to the Cy Dye Ester to the NHSester of the appropriate Cyanine fluor, Cy3-ester (Amersham Pharmacia, Cat# PA23001) and Cy5-ester (Amersham Pharmacia, Cat# PA25001). This was followed by purification of the two probes by passing through a Microcon 30 columns (Millipore, Bedford, MA) according to the manufacturer's instructions.

Hybridization

Hybridization occurred in 1× hybridization buffer containing 50% formamide, 5 × SSC, and 0.1% SDS. Prior to hybridization, the free amino groups on the slide were blocked or inactivated in the pre-hybridization solution containing 1% bovine serum albumin (BSA; Sigma Cat# A-9418), 5 × SSC and 0.1% SDS.

Data Collection

Primary data from image files were obtained using Scan Array Express II (Perkin Elmer, USA), a confocal laser scanner capable of interrogating both the Cy3- and Cy5-labeled probes and producing separate images

for each and then normalized using intensity and spatially dependent method (Yang *et al.,* 2002).

Following image processing, the data generated from the arrayed genes were further analyzed before differentially expressed genes could be identified. The first step in this process was the normalization of the relative fluorescence intensities in each of the two scanned channels. We calculated the normalization factors for each step of the experiment as follows: First, we used total measured fluorescence intensity in order to make the total mass of RNA labeled with either Cy3 or Cy5 equal. The total integrated intensity across all the spots in the array must be equal for both channels. Second, we used the scatter plot of Cy5/Cy3 of genes. (The scatter plot of Cy5/Cy3 of all genes is statistically examined). The scatter plots of the values of Cy3 and Cy5 fluorescent signals also revealed a pattern of distribution and were clustered in a diagonal line. A high correlation was observed in all samples and showed that there was a high reliability in the experiments by the cDNA microarray analysis of these samples. Third, we used some subsets of housekeeping genes that had already existed on each microarray chip. The ratio of measured Cy5 to Cy3 for these genes was modeled, and the mean of the ratio was adjusted to 1 (Zekri *et al.,* 2008).

We used Scan Array Express II (Perkin Elmer, USA) software for image processing. This software uses a threshold algorithm that separates spots from the background, allowing a grid to be laid across the spots. Having found a grid, spots are found within each grid element. The local background is calculated, and the background is subtracted. The integrated intensities were calculated for both the Cy3 and Cy5 channels. Measured intensities were analyzed using the Genesis software and R program that detect the up- and down-regulated genes according to the ratio in their software's (Zekri *et al.,* 2008).

Statistical Analysis

The results were analyzed using GraphPad Prism computer program (GraphPad software, San Diego, USA). For gene expression analysis, Mann-Whitney Test was used for numeric variables, and Chi square or Fisher's exact test was used to analyze categorical variables. The p value was considered significant when $P \leq 0.05$.

Results

Results of genes clusters affected by HCV infection

Apoptosis genes

We studied caspase genes as an example of apoptosis-related genes. CASP1 was downregulated more than 6 fold, CASP4 was downregulated more than 2 fold and BCL2 gene was upregulated more than 2 fold in HCV +ve group than in HCV -ve group. In contrast, the other caspase genes including CASP2, CASP5, CASP6, CASP7, CASP8, CASP9 and CASP10, as well as granzyme B gene showed no significant changes in the expression levels (Figure 1).

Genes Related to Leukemia or Lymphoma

We studied MLLLT3, BAL and lymphoid blast crisis genes. The microarray analysis showed that MLLT3 was upregulated more than 8 fold and BAL was upregulated more than 6 fold in HCV +ve group than in HCV -ve group. In contrast, the lymphoid blast crisis oncogene showed no significant changes in the expression level (Figure 2).

Transcription Regulator Genes

TATA box binding protein (TBP) was upregulated more than 5 fold in HCV +ve group than in HCV -ve group, in contrast, nuclear factor of κ light peptide inhibitor gene (NF.BIA), signal transducer and activator of transcription1 gene (STAT1) and transcription factor 3 gene showed no significant changes in the expression level (Figure 3).

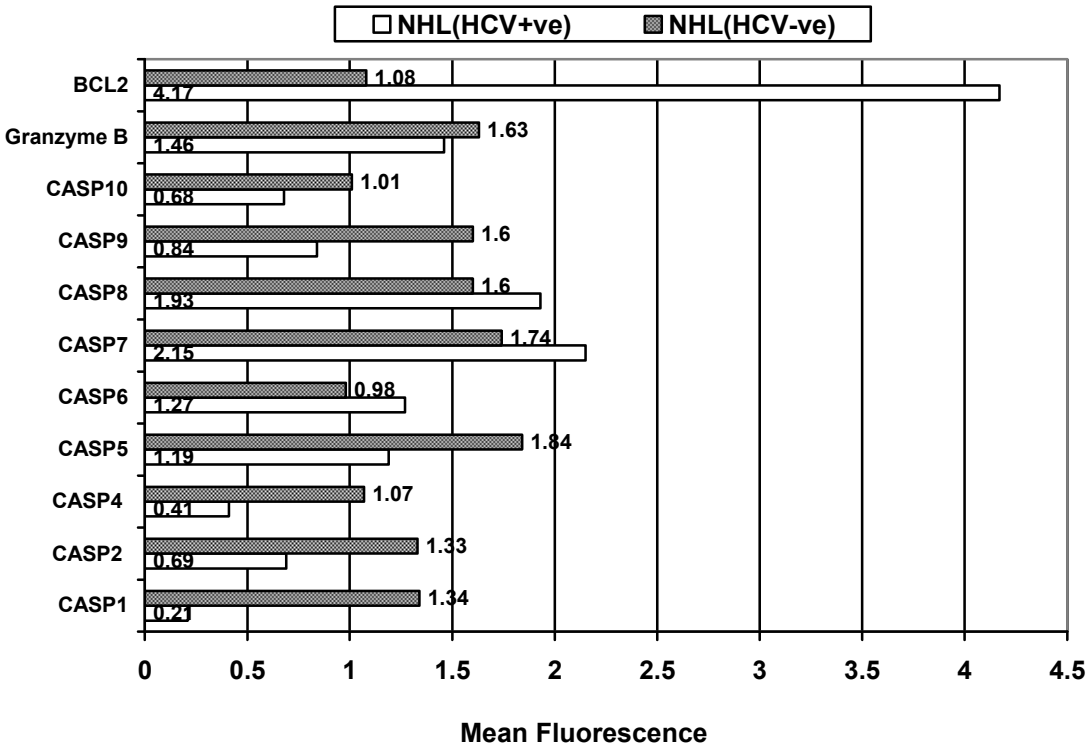

Figure 1. Gene Expression of Apoptosis Genes of HCV +ve & HCV -ve Groups.

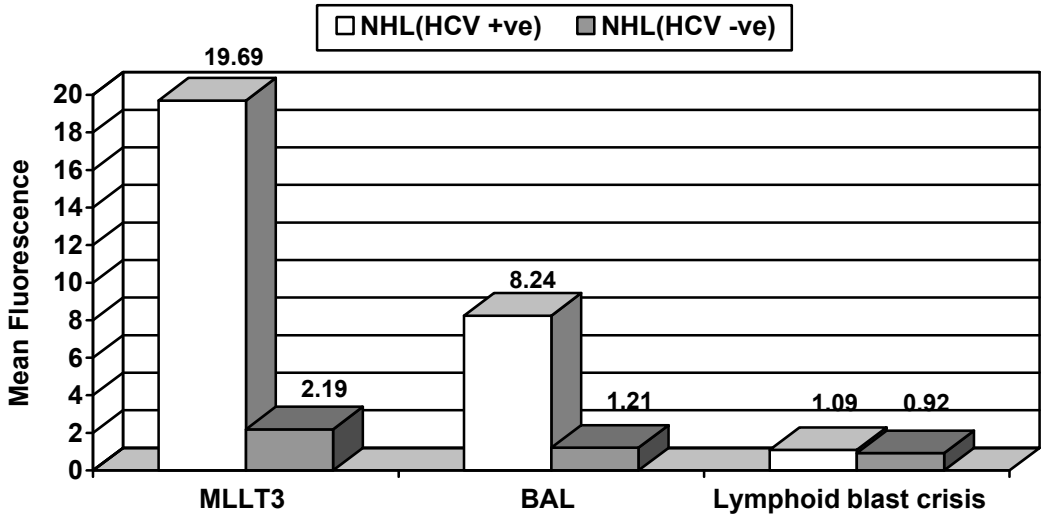

Figure 2. Gene Expression of MLLLT3, BAL and Lymphoid Blast Crisis Genes of HCV +ve & HCV -ve Groups

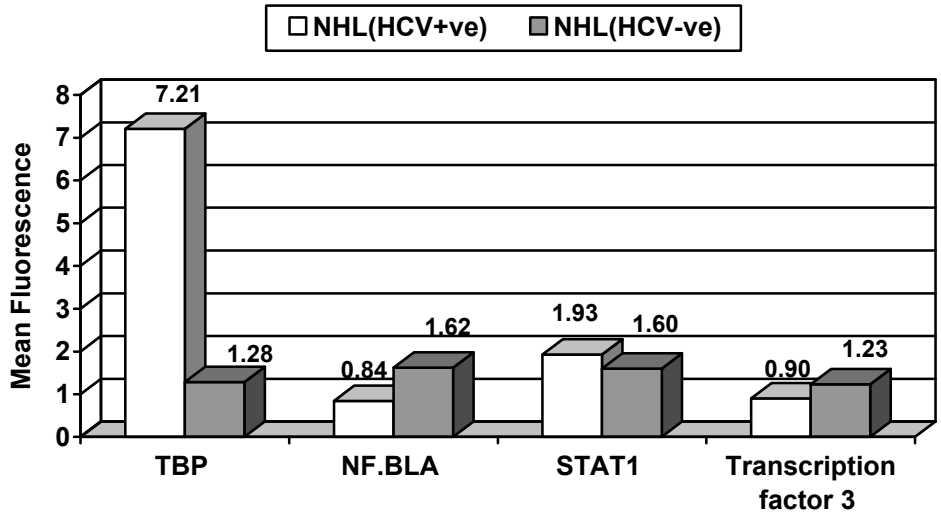

Figure 3. Gene expression of TBP & NF.BIA & STAT1 & transcription factor 3 genes of HCV +ve & HCV -ve groups

Immunoglobulin Genes

We studied gene expression of immunoglobulin superfamily containing leucine & Paired immunoglobulin genes. Immunoglobulin superfamily containing leucine gene was upregulated more than 4 fold in HCV +ve group than in HCV -ve group, in contrast, paired immunoglobulin gene showed no significant changes in the expression level (Figure 4).

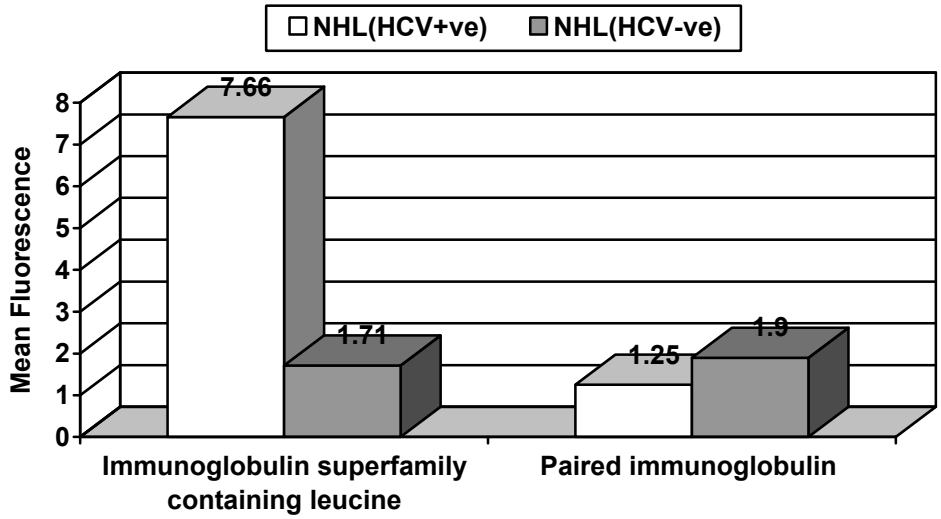

Figure 4. Gene Expression of Immunoglobulin Superfamily Containing Leucine & Paired Immunoglobulin Genes of HCV +ve & HCV -ve Groups

MHC Class II Molecules Genes

MHC class II, DM alpha gene (HLA-DMA) was downregulated more than 6 fold, MHC class II, DQ beta 1 gene (HLA-DQB1) was downregulated more than 5 fold, and MHC class II, DR alpha gene (HLA-DRA) was downregulated more than 3 fold in HCV +ve group than in HCV -ve group. In contrast, MHC class II, DM beta gene (HLA-DMB) showed no significant changes in the expression level (Figure 5).

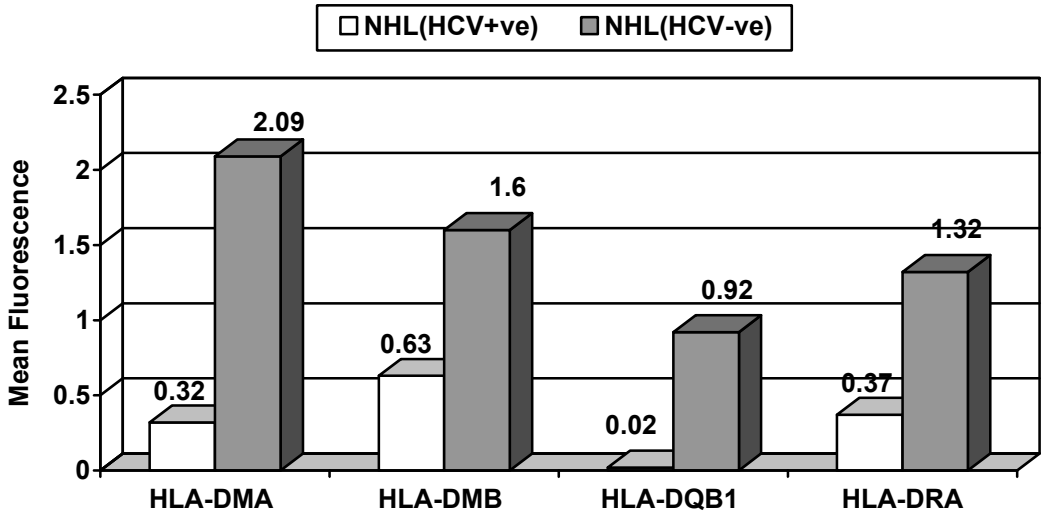

Figure 5. Gene expression of MHC class II genes (HLA-DMA & HLA-DMB & HLA-DQB1 & HLA-DRA) of HCV +ve & HCV -ve groups

Discussion

The molecular basis for viral induced B cell proliferation is still unknown (Ye *et al.,* 2003), genomic studies have been employed to investigate gene profiling associated with hepatitis virus infection, thereby providing more insights into the molecular mechanism of viral infection (Wu *et al.,* 2002). More recently, a number of microarray analyses have been performed in chimpanzees and in patients infected with HCV virus in an attempt to identify specific gene expression profiling and potential markers (Bigger *et al.,* 2004).

In this study, we performed microarray analyses for gene expression profiling in patients with B-cell non-Hodgkin's lymphoma with HCV infection to examine the molecular effects of HCV on regulating gene expression in B-cell non-Hodgkin's lymphoma using microarray chips covering nearly 15,500 human oligonucleotides, more than 1000 differentially expressed genes were clustered based on significance (P value < 0.05). Most genes appeared to be either upregulated or downregulated, indicating that HCV can modulate cellular gene expression in B lymphocyte in several clusters of genes. The identified genes could provide a new gate for prognostic and diagnostic markers for NHL associated with HCV infection. They could also be used to identify candidate genes for molecular target therapy.

Apoptosis-related genes are a large group of genes associated with programmed cell death as caspase genes, granzyme genes and BCL2 gene (gene of anti-apoptotic Bcl-2 protein), HCV may rescue B lymphocytes from apoptosis possibly through causing suppression of CASP1 and CASP4 and causing overexpression of the anti-apoptotic BCL2 gene. In the current study, CASP1 was down-regulated more than 6 fold; CASP4 was downregulated more than 2 fold in HCV +ve group than in HCV -ve group. A previous study on the effect of HCV core protein on the molecular profiling of human B lymphocytes was reported by Wu *et al.* (2006) showed near results, CASP1 and CASP4 were down-regulated more than 2 fold in B cells infected with HCV core protein.

Apoptosis may involve binding of HCV core protein to the intra-cellular signal transducing portion of death receptors and displacement of signaling molecules. Hence, monitoring caspase activation might provide a reliable tool to estimate the efficacy of HCV therapy, and might open challenging therapeutic strategies in HCV infection (Castelli *et al.,* 1997).

BCL-2 is a human proto-oncogene located on chromosome 18. Its product is an integral membrane protein (called Bcl-2) located in the membranes of the endoplasmic reticulum (ER), nuclear envelope, and in the outer membranes of the mitochondria. The gene was discovered as the translocated locus in a B-cell leukemia (hence the name), this translocation is also found in some B-cell lymphomas.

In agreement with our results, the portion of chromosome 18 in the malignant B cells containing the BCL-2 locus has undergone a translocation with the portion of chromosome 14 containing the antibody heavy chain locus. This t(14;18) translocation places the BCL-2 gene close to the heavy chain gene enhancer, this enhancer is very active in B cells (whose job it is to synthesize large amounts of antibody). So it is not surprising to find that the Bcl-2 protein is expressed at high levels in these t(14;18) cells (Zuckerman *et al.,* 2001).

In addition, a previous study showed that there is overexpression of the anti-apoptotic Bcl-2 protein with a higher Bcl-2/bax ratio in t(14;18)-positive B-cell samples, as well as a modification of detectability of t(14;18) B-cell clones following antiviral treatment (Giannelli *et al.,* 2003).

Several genes related to myeloid/lymphoid leukemia and B lymphoma were among the most expressed genes of HCV infection as MLLT3, BAL and lymphoid blast crisis oncogene. MLLT3 is involved in translocations associated with both acute lymphoblastic and acute myelogenous leukemia (Shago *et al.,* 2004). Also, BAL (B-aggressive lymphoma) is a novel risk-related gene in diffuse large B-cell lymphomas that enhances cellular migration. We found overexpression of as MLLT3, BAL genes in HCV infected cases.

A previous study reported similar results with higher expression levels, MLLT3 and BAL genes were upregulated >10 folds (Wu et al., 2006), also a previous study showed that stable BAL-over expressing B-cell lymphoma transfectants had significantly higher rates of migration than vector-only transfectants, indicating that the risk-related BAL gene promotes malignant B-cell migration (Aguiar *et al.,* 2000).

We observed overexpression transcription regulator genes as TATA box binding protein (TBP) associated with HCV. A previous study on the effect of HCV core protein on the molecular profiling of human B lymphocytes showed near results, TBP and NF.BIA were upregulated more than 4 fold in B cells infected with HCV core protein. But in controversy to our results this study showed downregulation of the STAT1 gene (Wu *et al.,* 2006).

We also found overexpression of the immunoglobulin superfamily containing leucine gene in association with HCV. HCV is frequently associated with mixed cryoglobulinemia (Ramos-Casals *et al.,* 2005; Saadoun *et al.,* 2005), raising the possibility that HCV may influence the regulation of immunoglobulin genes in B cells. These results suggest that overproduction of immunoglobulins in B-lymphocyte disorders may be associated with HCV infection. Alternatively, other cell types, for example, T cells or macrophages, may activate B cells and eventually lead to the overproduction of immunoglobulin in these patients.

Wu *et al.* (2006) found that none of the immunoglobulin genes showed any significant changes in transcription levels, but contrasting data, showing that HCV may favor mutations of immunoglobulin genes and oncogenes by a “hit and run” mechanism, have recently been obtained both in cell lines and in cultured cells taken from HCV-infected patients (Machida *et al.,* 2004).

In the current study, HCV infection was associated with reduced expression of MHC class II molecules in B lymphocytes. Therefore, inhibition of antigen processing and presentation through downregulation of different MHC class II molecules genes, thus, it is possible that HCV modulation of MHC class II expression and antigen presentation to CD4+ T cells provides HCV with a means of avoiding early immune recognition (Wu et al., 2006). Our finding is in agreement with a recent study found that all MHC class II molecules including HLA-DMA, HLA-DMB, HLA-DQB1, and HLA-DRA, together with CD74, an MHC class II molecule related gene, were dramatically suppressed more than 10 folds (Wu *et al.,* 2006).

Also, our findings are in agreement with a previous study demonstrating reduced expression of MHC class II molecules in human cytomegalovirus-infected macrophage cultures from 90% of the donors (Odeberg & Soderberg-Naucler, 2001). Human cytomegalovirus uses different mechanisms to decrease the expression of MHC class II molecules on infected macrophages either independent of or dependent on viral replication. Moreover, CMV-infected macrophages exhibited a 66% to 90% reduced capacity of stimulating an antigen-specific proliferative CD4+ T-cell response (Odeberg & Soderberg-Naucler, 2001).

It is generally accepted that genes within the major histocompatibility complex play a central role in the development of the immune response against HCV. MHC class II molecules may be important for viral clearance, because particular alleles are associated with chronic HCV infections in patients, and MHC class II molecules restrict HCV-specific CTL responses (Yenigun & Durupinar, 2002;, McKiernan *et al.,* 2004), furthermore, inhibition of MHC class II molecules, and therefore inhibition of antigen processing and presentation, may account for delayed development of neutralizing antibodies against HCV, as observed both in animal experiments and in HCV-infected patients (Soguero *et al.,* 2002; Yao *et al.,* 2004).

Future studies directed at the mechanism of suppression of MHC class II molecules and regulation of T cell function by HCV may identify new approaches for therapeutic regulation of HCV infection.

We conclude that the upregulated and the downregulated genes identified through the studied expression profiles of NHL with HCV infection may shed light on the mechanisms of HCV lymphomagenesis. Further studies are needed to assess the multifactorial process of lymphomagenesis in which genetic, environmental and infectious factors seem to interplay and to identify candidate genes for molecular target therapy.

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