CHAPTER-5

DISCUSSION
Hepatocarcinoma is a major health problem, both in developed as well as in developing countries. According to World Health Organization, HCC is the third leading cause of cancer-related deaths, exceeded only by cancers of the lung and stomach (WHO report, 2008). The incidence in the western countries is rising, mainly because of chronic liver injury, chronic viral hepatitis and alcoholic liver disease (Bruix et al., 2004; Llovet et al., 2004). The highest incidence of HCC is found in areas where the hepatitis B virus (HBV) and hepatitis C virus (HCV) are endemic. About 80% of people with hepatocellular carcinomas have liver cirrhosis (Kamel and Bluemke, 2002). The survival rate after the onset of symptoms is generally less than one year (Tan et al., 2009). The absence of specific clinical symptoms at early stage and paucity of curative treatment for late stage are two important factor that contribute to the high mortality (Tan et al., 2009). Thus, there is an urgent need to develop new therapeutic targets and to identify novel biomarkers for the early detection of HCC.

Proteomic studies provide the analysis and evaluation of complete set of proteins expressed in a given cell, tissue or biofluid, characterizes protein expression profiles and also identifies protein structures, localizations, activities, modifications and interactions in physiological or pathological states. As proteins perform majority of the biological functions, proteomics bridges the gap between the information coded in the genome sequence and cellular behavior. Liver carcinogenesis is a multistep process characterized by the accumulation of successive molecular genetic and epigenetic alternation, structural aberrations including deletions and translocations resulting in selection of clonal cells with uncontrolled growth capacities. Proteomic is a novel approach to understand the tumorigenesis events of HCC (Feng et al., 2006).

5.1 DEVELOPMENT OF MODIFIED HCC MODEL
Carcinogenesis process is accompanied by fundamental changes in the genome, which further lead to many alterations in the cellular phenotype (Goldfarb and Pugh, 1986; Moore and Kitagawa, 1986). Several in vivo models have been developed to study multistage carcinogenesis in the liver (Solt et al., 1977; Goldsworthy et al., 1986; Dragan et al., 1997; Tamano and Shirai, 2003).
However, in all the reported models, the initiation can be brought about either by administering a necrogenic dose of a hepatic carcinogen or by administering a lower dose of a carcinogen in combination with partial hepatectomy. For administering weak agents or determining if an unknown chemical has the initiating activity, the use of partial hepatectomy is desirable for increased sensitivity. Partial hepatectomy, however, requires extensive surgical procedure and can be extremely painful to animals. Present study shows that the need for partial hepatectomy could be eliminated with alternative method.

We have used rodents as animal of choice as their relatively short life span permits the observation of cellular transformation from its initiation to full-blown malignant cancer. A modification was made in the Solt and Farber protocol (Solt and Farber, 1991). Hepatotumorigenesis was developed in male Wistar rats by administering of single interperitoneal (i.p) dose of DEN (200 mg/kg b.wt) and repeated oral doses of 2-AAF (150 mg/kg b.wt; Figure 4.1) (Malik et al., 2012). The method is novel as it eliminated both the need for necrogenic doses of carcinogen as well as that of PH. A variety of clinical alternations/symptoms were seen within 30 days of DEN+2-AAF administration. These included loss of hair, blood clotting problems, loss of appetite and water intake. Loss of hair started at day 20 (Figure 4.2) and the amount of food intake was became lowered by up to one half after 90 days of DEN+2-AAF administration. It was also observed that rats that received carcinogens showed a significant increase in their liver weights while their body weight was decreased with the progression of disease (Table 1). The increase of liver weight is caused by small tumors and well developed liver nodules. The decrease of body weight observed in hepatoma is a common symptom in malignant tumors (Ha et al., 2001). While the incidence of preneoplastic foci and enlarged liver were not significant at day 30 in the DEN+2-AAF administrated group, on day 90-120 the incidence of nodules was in almost 100% cases in the treated groups (Figure 4.3). DEN induced the transformation of hepatocytes, and the transformed cells were able to grow faster when challenged with another carcinogen (2-AAF) that served as promoter. The 2-AAF administration is known to inhibit the proliferation of normal or surrounding hepatocytes but increases the growth of DEN altered
hepatocytes (Solt and Farber, 1991). DEN showed similar effect as showed by aminoazo dyes and developing necrosis, hyperplasia, hyperbasophilia and tumor.

The results were further supported by histopathological changes of the liver that confirmed the cancer development and its progression. DEN+2-AAF administration initially induced hepatocellular degeneration around the central lobule in the liver tissue, and these degenerated liver cells then became the precursors of hepatoma as confirmed by histopathological observation. These precursors are called neoplastic tubercules, degenerated lesions, and proliferative tubercles (Farber, 1987). Dunsford et al. (1989) reporting on hepatocellular vacuolization in animals administered with DEN observed that vacuolar liver cells appeared from 5 weeks onwards and increased in size and number up to 9 weeks after treatment. In our animal model, the hepatocellular vacuolization was also observed as a small number of surrounding hepatolobules at day 30; and hepatocellular vacuolization developed in almost all liver cells, resulting in necrosis that progressed to full adenomas at 120 days with details of the adenomatous nodules in the liver parenchyma. At day 60, only bile duct proliferation was observed in large area while large nodules in the liver parenchyma were prominent at day 90. The cells in the nodule were large with clear cytoplasm without sinusoidal spaces. At 120 days, big adenomas were present in liver parenchyma and clear nuclear atypia was seen in the adenoma (Figure 4.4).

These observations are the agreement with reported changes in various types of liver cancers (Ha et al., 2001). No such cell damages were observed in the control groups. At the time of sacrificing, size of the liver tumors was 0.2 to 0.3 mm while other major organs such as kidney and lung were found to be normal at this stage. The type of slow progression of tumor formation provided sufficient time to analyze significant changes in sera at proteome level (Malik et al., 2012).
5.2 ASSESSMENT OF LIVER FUNCTION TEST AND BIOCHEMICAL ENZYMES

The activities of hepatospecific enzymes are altered when hepatocellular damage leads to abnormalities of liver function. It has been reported that ALP, AST and ALT exhibit higher activity in abnormally functioning liver, and are routinely used as marker for proper functioning of liver. These have also been used as index of liver function recovery degree in liver transplant patients (Simonsen and Uirji, 1984; Alwahaibi et al., 2010).

In the present study, ALP activity was increased significantly in treated rats and showed a correlation with the progression of disease (Figure 4.5A). We also found elevated levels of AST and ALT in treated rats sera as the disease progressed (Figure 4.5B & C). These results support the hepatocellular degeneration by DEN+2-AAF administration and are in agreement with earlier reports (Whittby et al., 1984). A significant increase in ALP, AST and ALT enzyme levels in serum has also been reported after inducing hepatocellular tumors by administering CCl4 in rats (Kim et al., 1994). In the liver, ALP is closely associated with lipid membrane in the canalicular zone, so that any interference with the bile flow, whether extra-hepatic or intra-hepatic leads to increased serum levels of ALP activities.

DEN causes oxidative stress and cellular injury due to the enhanced generation of reactive oxygen species (ROS) (Bartsch et al., 1989). The free radicals generated by the enzymes of mixed function cytochrome P450-dependent monoxidase system may augment the oxidative stress by the formation of H2O2 and superoxide anions (Farber and Gerson, 1984). ROS are highly dangerous byproducts of cellular metabolism that have direct effect on development and growth of the cell and its survival on the development of cancer. As liver is the main site for metabolic biotransformation of DEN, the production of ROS in liver may be responsible for oxidative stress which causes liver damage (Gey, 1993). The cellular damage caused by ROS can easily be measured in terms of lipid peroxidation (LPO; Spiteller, 1996). Such findings corroborate with our results showing elevated level of malondialdehyde (MDA) in DEN+2-AAF treated rats, bearing a correlation with progression of disease (Figure 4.6A). The activity
of the antioxidant enzyme SOD in serum of DEN+ 2-AAF treated rats was found to be significantly decreased (Figure 4.6B). Decreased activity of SOD has been reported in pathological conditions (Halliwell and Gutteridge, 1989). In present study, the decrease in catalase activity (Figure 4.6C) may have relationship with excess H₂O₂ production following DEN+2-AAF treatment or due to SOD inhibition. Hence it is assumed that the decrease in activities of oxidative stress enzymes might be due to the increased free radical attack i.e. due to elevated lipid peroxidation products (MDA) in sera of cancer patients.

5.3 APPLICATION OF PROTEOMICS FOR THE SEARCH OF NOVEL TUMOR SPECIFIC PROTEINS

Serum contains a large number of secreted or shed low abundance proteins that are critical for signaling cascades and regulatory events. In the present study, we compared the protein profiles of the sera of control and liver cancer induced rats using 1D, 2-DE and LC-MS/MS approaches. Using this approach we have observed a number of proteins that were differentially expressed in control and treated rat sera. The changes in protein profiles have been analyzed at different stages of disease, starting from the initiation to full blown tumor development. Serum samples from both control and treated rats were processed to selectively deplete one of the most abundant proteins, albumin which account for nearly half of the total serum protein content and same quantity of proteins was run on SDS-PAGE (Figure 4.7). Clearly defined bands indicated that protein samples are intact. Some differentially expressed proteins were seen. However, for better resolution it was thought pertinent to analyze the proteins by two dimensional gel electrophoresis which can further resolve proteins.

Two dimensional gel electrophoresis (2DE) has evolved as a robust method to characterize the proteome. This technique uses IEF and SDS-PAGE in perpendicular directions. Broad pH range (pH 3-10) was used for 2DE (Figure 4.8). The proteins were maintained in a fully reduced state. Detergent in RBs prevented protein interaction and aggregation, and their properties are critical for protein solubilization. The addition of carrier ampholytes enhances solubility of individual proteins as they approach their isoelectric points. They also produce
an approximately uniform conductivity across a pH gradient without affecting its shape. The focusing time was increased as it helped in better resolution during electrophoresis. The duration of the IEF step was dependent on both sample conductivity and protein loading, producing good results if performed for a total of more than 33,000 volts-hours. The actual voltage was limited since the current was restricted to 50 μA/strip. This total value of volt-hours was enough to complete focusing in samples with the highest carrier ampholyte concentrations.

After IEF, the focused gel was prepared for SDS-PAGE, usually by incubating consecutively in two equilibration buffers containing DTT or iodoacetamide (IAA) respectively. IAA serves to alkylate reduced cysteine residues and prevent their modification during and after SDS-PAGE. Three different sets of experiments have been carried out to compare the pattern of cellular protein expression in control and DEN+2-AAF treated serum samples and among these three pairs, one was chosen for PD-Quest analysis (Figure 4.9). PD-Quest software compares computer images of 2D gels to determine differential protein expression. Modern day 2-DE research often utilizes software-based image analysis tools. These tools primarily analyze bio-markers by quantifying individual proteins, and showing the separation between one or more protein "spots" on a scanned image of a 2-DE product (Payungsak et al., 2005). PD-Quest software automatically selects spot, cuts in order from the least to the highest quantity of protein. A total 46 proteins spots were observed in the analysis of 2D-gels among which 14 proteins (A-N) were differentially expressed between control and treated sample. 1-28 proteins were expressed only in control while 4 proteins (29-32) were only expressed in carcinogen treated animals (Table 2). Additionally, these tools match spots between gels of similar samples to show proteomic differences between early and advanced stages of an illness (Payungsak et al., 2005).

In the present study, we observed a number of proteins that were differentially expressed in control and DEN+2-AAF treated rat sera. Four of these proteins that showed significantly differential expression (Figure 4.11), were characterized by LC-MS/MS and identified as Transthyretin precursor, Complement Component C3 (Spot G, Molecular weight: 15.82, 32.41 kDa, pI: 115
5.77, 5.73, mouse score: 89, 87), Apolipoprotein AI precursor (Spot I, Molecular weight: 29.95, pI: 5.45, mouse score: 80), Immunoglobulin gamma 2A chain C region (Spot K, Molecular weight: 35.67, pI: 7.72, mouse score: 89) and Immunoglobulin J chain precursor isoform 2 (Spot N, Molecular weight, 18.22: pI: 5.18, mouse score: 106) (Figures 4.12 - 4.15; Table - 3)

5.3.1 COMPLEMENT COMPONENT C3

Tumor markers are secreted, released or leaked into the interstitial fluids, and thus into the lymph, and finally (or directly) into the bloodstream, where they become detectable in serum samples. As IgG 2A chain C region and Immunoglobulin J chain precursor isoform 2 are commonly expressed in most of the diseases, they cannot serve as reliable and disease specific biomarkers. It was therefore, decided to select the other three proteins (complement C3, apolipoprotein AI precursor and transthyretin precursor) for further studies. The expression of these proteins with the progression of disease over the period of 120 days was studied. It was found that the expression of complement component C3 is elevated in the early phase (15 days or earlier) of the disease and then the levels remain elevated throughout the study period without any further increase in its concentration. Complement C3 is the major plasma protein of the immune system and complement system is one of the most important weapons of innate immunity which is involved in all infectious processes (Malik et al., 2012). It is not only a mechanism for direct protection against an invading pathogen but it is also involved in the adaptive immunity and optimizes the pathogen specific humoral and cellular defense cascade in the body (Speth et al., 2003). The C3 protein is essential for activating the complement system. The presence of foreign invaders triggers the C3 protein to be cleaved into two smaller pieces. One of these pieces, called C3b, interacts with several other proteins on the surface of cells to trigger the complement systems response. In cancer patients, complement activation with subsequent deposition of complement components on tumor tissue have been reported (Neng et al., 2006). The levels of circulating immune complexes and complement become significantly higher in HCC patients (Takezaki et al., 1990). The elevated levels of complement component C3a have
been predicted to be candidate marker of chronic hepatitis C and HCV related hepatocellular carcinoma (Lee et al., 2006). In HCC patients, increase in C3 and C4 were found to be good diagnostic tools for HCC screening in liver cirrhosis patients (Roba et al., 2007). We studied the complement C3 expression levels at different time interval (15, 30, 60, 90 and 120 days respectively post treatment; Figure 4.16A). The findings of the study clearly demonstrate that complement C3 protein expression level were elevated at very early stage even before the full blown tumor development. Moreover, the elevated levels of complement C3 in chemically induced liver cancer animals remained almost same with disease progression (Figure 4.16B). These observations show the potential of this protein as an early biomarker. The C3 protein expression levels were also analyzed in human cancer patient sera when similar elevated levels were obtained. This further validated the up-regulation of this protein (Figure 4.19 A). This protein may therefore represent a powerful tool in search for a candidate biomarkers for early detection of HCC.

5.3.2 APOLIPOPROTEIN AI PRECURSOR (PROAPO AI)

Another protein that was identified in the DEN+2-AAF treated rats sera was proapo AI. Liver is the main organ for the synthesis, storage, transportation and degradation of several apolipoproteins. Apolipoprotein AI (apoAI) is the major protein constituent of high density lipoproteins (HDL) and lymph chylomicrons (Kontush and Chapman, 2012). In human, apo AI is secreted into the plasma as proapo AI, with the prosegment consisting of a 6-amino acid NH2-terminal extension (Zannis et al., 1983). After secretion into the plasma, this prosegment is cleaved by a protease named proapoAI converting enzyme, which converts the proapo AI to the mature apoAI extracellularly (Edeistein et al., 1983; Gordon et al., 1983; Zannis et al., 1983; Bojanovski et al., 1985). Higher level of proapoAI was reported in the plasma of patients with liver cirrhosis than in normal subjects (Suehiro et al., 1986, Matsuura et al., 1988). Despite the determination of its characteristics, the role of this protein on lipoprotein metabolism is not yet fully understood. Some apolipoproteins have been implicated as serum markers in different types of cancer (Zhang et al., 2004). It was reported that risk of HCC
increases when there is high level of this protein in plasma or serum (Maria et al., 2010). In our study, proapoAI is up-regulated in liver cancer; the levels peaked on day 30 and remained elevated during the disease progression (Figure 4.17A, B). Its high level may be the result of impairment in the activity of the proapoAI converting enzyme (Isobe et al., 1990). Further confirmation of this protein was done in human cancer patient sera which also showed its elevated levels (Figure 19B). The biological and clinical implications of the protein as metabolic marker therefore, need to be further investigated.

5.3.3 TRANSTHYRETIN PRECURSOR

Transthyretin (Prealbumin) is a plasma protein involved in the transport of retinol and thyroxine. Liver is the main site of transthyretin synthesis (Felding P and Fex G, 1982). Transthyretin is a sensitive marker of metabolic stress and in cancer patients it has gained considerable interest with regard to the use as an early diagnostic marker (Beat et al., 2005). Transthyretin and its modified forms were identified by direct mass-spectral profiling and have been proposed as probable biomarkers of ovarian cancer (Kozak et al., 2005), B-cell-lymphoma (Miguet et al., 2006), colon cancer (Fung et al., 2005), pancreatic cancer (Ehmann et al., 2007), mycosis fungoides (Escher et al., 2007), lung cancer (Goufman et al., 2006; Cho, 2007) and cholangiocarcinoma (Liu et al., 2008). In our study this protein has been found to be over-expressed in serum samples of DEN+2-AAF treated rats in comparison to control. The levels reached to maximum at day 30 and remained elevated at different time interval (Figure 4.18A, B). Due to limited literature about the function of transthyretin expressed in liver cancer cells, it is still a question whether cancer derived transthyretin has similar function as normal transthyretin or it has an altered role. Further, the modified derivative of this protein could be cancer specific (see later-section 5.4). The transthyretin expression levels were also analyzed in human cancer patient sera when similar elevated levels were obtained. This further validated the up-regulation of this protein (Figure 4.19 C). More studies are needed to answer a number of key questions regarding this observation. The molecular mechanism, biological function and clinical significance of transthyretin production in liver
cells, particularly cancer cells, warrants in-depth investigation. It would be of interest to examine whether transthyretin expression can be used as a diagnostic and prognostic marker or a therapeutic target. It is likely that the answers to these questions will give us new clues about carcinogenesis and cancer therapy.

5.4 CLONING AND EXPRESSION OF TRANSTHYRETIN PRECURSOR
The protein (transthyretin precursor) showing the maximum MOWSE score was chosen to further validate its corresponding gene expression in DEN+2-AAF induced liver cancer tissue by RT-PCR analysis. This study was designed to evaluate transthyretin gene expression and its possible significance in liver tissue samples. Total RNA was isolated from liver by TRI reagent. Two clear bands of ribosomal RNA (28s and 18s) were visible, showing the RNA isolated was pure and intact (Figure 4.20). cDNA sequence analysis for the protein revealed an open reading frame of 444 bases that resulted in a protein of 147 amino acids (Figure 4.21). Using the gene sequence data, oligonucleotide primers were designed and full length cDNA was amplified by RT-PCR (Figure 4.22). The PCR amplified fragment was purified to remove free primers as these may reduce the efficiency of ligation of PCR product to vector during subsequent steps (Figure 4.23, 4.24). The ligation mixture was used for transformation of E. coli DH5α cells. The cells were screened for the presence of cloned gene on the basis of blue-white screening (Figure 4.25). As the T vector carries ampicillin resistance gene and only the cells carrying plasmid would be able to survive ampicillin selection pressure. The positive colonies were picked from the culture plate and identified by colony PCR using insert specific primers (Figure 4.26). The transformants were checked for the presence of the insert by plasmid isolation and double digestion with Neo I and Bam HI showing the release of 444 bp fragment (Figure 4.27). Further confirmation of r-clones was done by sequence analysis using forward primer. The transversion of four bases were found in the cloned sequence (Figure 4.28). The alignment and comparison of the sequence of gene showed 99% homology with the reported sequence of transthyretin (Figure 4.29). The nucleotide sequence was translated to deduce the amino acid sequence of the protein. The comparison of the amino acids sequence with rat transthyretin
sequence showed 98% homology (Figure 4.30). There were changes in three amino acids. These changes may represent a modified tumor specific protein (transthyretin). Being a tumor specific protein, it was expected that there may be an immune response to this protein.

pET-32(a) vector was used for expression of transthyretin. This vector is designed for high-level prokaryotic expression controlled by the strong bacteriophage T7 promoter/lac operator. Expression is induced by the production of T7 RNA polymerase in BL21 (DE3) E. coli cells (Figure 4.31). The 444 bp gene was subcloned in vector pET-32(a) between Nco I and Bam HI sites in MCS region. The vector and PCR product were double digested with Nco I/Bam HI enzymes, ligated and yielding the expression construct pSJ-6.3kb (Figure 4.32). As the cloning was done in directional manner there was no need to check the orientation. Therefore, the ligation mixture was directly transferred into E.coli BL-21 (DE3) cells. The cells were screened for the presence of cloned gene on the basis of antibiotic selection pressure (ampicillin) (Figure 4.33). The positive cells were picked up from the culture plate and colony PCR was done to check the presence of insert. Colonies number 1, 3 and 6 showed strong amplification of r-clones (Figure 4.34). Further, to confirm the presence of r-clones double restriction digestion (Nco I/Bam HI) of recombinant plasmids was done to pop out the insert (Figure 4.35). The recombinant plasmids that gave strong positive signals were selected for expression studies. In the resulting construct pSJ-6.3, the inserted gene is fused in frame with ATG codon (5'end) and 6x His tag coding sequence (at 3'end). For protein expression, cultures were incubated at 37°C until the absorbance at 600nm reached to 0.6-0.8 before adding IPTG (1mM) for induction of recombinant protein expression.

It was evident from a comparison of the polypeptide profiles obtained in the absence and presence of IPTG that induction of gene expression results in the appearance of a new (approximately 17 kDa) band, which is consistent with the predicted size of recombinant protein (Figure 4.36). IPTG, an inducer of lac repressor-regulated promoters was used to induce the lac operon because, in contrast to allolactose, which is the natural inducer of the operon, IPTG cannot
be hydrolyzed and broken down by the cell. Hence its concentration does not change during an experiment (Bhat and Jain, 2010). In case of *E. coli* BL21 (DE3) system, addition of 1 mM IPTG was found to be optimal for showing an increase in the expression of 17kDa gene. An increase in the IPTG concentration from 1 mM did not show any significant improvement in the expression of 17kDa protein.

To obtain functionally active protein, 17 kDa induced protein was purified from *E. coli* via the 6xHis tag expressed on the carboxy terminus of the protein using nickel-NTA chromatography. Urea was included in the lysis buffer to solubilize the proteins. Solubilization is a critical step towards obtaining maximal amount of the desired protein in solution without inducing any chemical or deleterious modifications to it. Wide panels of detergents are available for the solubilization of inclusion bodies including strong denaturants like urea, guanidinium salts and detergents such as sodium dodecyl (Stockel *et al.*, 1997), n-cetyl trimethylammonium bromide (CTAB) (Cardamone *et al.*, 1995) and sarkosyl (Burgess, 2009). In our study buffers of different pH and urea as denaturant were used for solubilization. Usually, an intermediate concentration (Urea-8M) of the denaturant is taken, which is low enough to force protein molecules to collapse, yet can allow them to stay in solution and be flexible to reorganize their structure. This process results in the single-step purification of the tagged protein to yield a very pure, almost homogenous sample (Figure 4.37). The purified fractions were analyzed by SDS-PAGE (figure 4.38). Approximately 80–90% of the induced protein bound to the column and it also appears that there was no significant non-specific binding to the affinity matrix. Elution with a pH 4.5 buffer resulted in the emergence of highly purified (> 95%) recombinant protein. Lowering the pH (to 5.3–4.5) of elution buffer protonates the imidazole nitrogen atom of the histidine residue. This results in disruption of the coordination bond between the histidine and the transition metal, and the desired protein easily separate out from the column (Joshua *et al.*, 2010). Thus it was possible to purify the r-proteins to near homogeneity by a single step Ni-NTA affinity chromatography with a yield of approximately 40 mg/litre of culture.
To validate the presence of antibodies against the recombinant protein transthyretin in rat sera, Western blot analysis was carried out. It was observed that the antibodies for transthyretin were present in treated rat sera at 15 days and increased with progression of cancer when compared to the control group. No such antibodies were found in control animals (Figure 4.39). This suggests that immune system of the animal responds to modified transthyretin protein and its up-regulation in cancer might have triggered the formation of antibodies detectable in serum of DEN+2-AAF treated rats, thus invoking the humoral response in them (Wu et al., 2009). The higher level of a self protein may also produce auto antibodies under pathological condition. It has been reported auto antibodies associated with several types of cancers including, cancers of the liver, breast, lung, head and neck, colon, ovary and prostate, offering the potential of improved diagnosis for these cancers (Wang et al., Gnjatic et al., 2009; Curtis et al., 2012). The immune response occurs early during tumor development and as a result the presence of autoantibodies against tumor antigens in serum might provide an effective means for cancer screening and early diagnosis (Tan and Zhang, 2008; Qiu and Hanash, 2009). It is believed that autoantibodies are generated through over-expression, mutation, release of proteins from damaged tissues, mis-folding or mis-presentation of proteins which leads to their recognition by the immune system. Modification of the gene leading to accumulation of its expressed protein due to increased stability can be measured through immunoassay. The accumulated protein then acts as an antigen, with subsequent development of antibodies which are detectable in tissues, sloughed cells, blood and other body fluids.

Our study suggests that the presence of tumor-derived transthyretin in liver cancer might be contribute in cancer initiation in the precancerous stage when the epithelial cells are actively proliferating. HCC cell produced modified transthyretin which is involved in the biological behavior of this cancer and may serve as useful marker for cancer cell differentiation and prognosis. Thus, the determination of elevated level of transthyretin by itself or in combination with conventional markers may provide relevant information regarding the noninvasive early stage detection of hepatocellular carcinoma.