CHAPTER-6

SUMMARY

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CONCLUSIONS
Hepatocellular carcinoma is an international problem. It is third most common cancer and the fifth leading cause of cancer death worldwide (WHO-2008). The current standard diagnosis of HCC realizes on detection of the serum α-fetoprotein (AFP) level. AFP marker is well received for its low sensitivity (64.8% - 78%) and specificity (50% - 93%). To this end reliable and accurate biomarker are urgently needed to overcome the shortcoming of the current methodology of HCC diagnosis. Proteomic is a novel approach to study the biological system by qualitative and quantitative analysis of all the proteins present in a cell type. This approach opens a new way for discovering novel biomarker(s) that can be used to diagnose, predict susceptibility and monitor progression of diseases. The human serum proteome (secretome) can be non-invasively measured and it provides a tremendous opportunity for detecting, therapeutic monitoring, and deciphering basic cancer mechanisms.

Based on the results of our studies the important conclusions drawn are summarized as under:

- A modified non-surgical method for induction of liver cancer in Wistar rats using a combination of DEN+2-AAF has been developed.
- Validation of successful development of rodent model was made by histopathological observations of the liver tissue.
- Liver function enzymes were studied at different time interval to check the liver abnormalities. It was found that LPO was elevated by 84.42%, SOD and CAT activity was decreased by 71.50% and 76.60% respectively in serum of DEN+2-AAF treated rats when compared with control.
- Serum specific protein expression profiles in control and treated animals have been studied using 1D and 2D gel electrophoresis. A number of proteins ranging in molecular weight from 20 kDa to 66 kDa were found to be differentially expressed between the control and the treated animals.
- PD-Quest software analyses of 2-DE gels showed total 14 differentially expressed proteins, among which 4 significantly differential proteins spots were characterized by LC-MS/MS and identified as Transthyretin precursor
and Complement C3 (Spot G), IgG 2A chain C region (Spot K), Immunoglobulin J chain precursor isoform 2 (Spot N), and Apolipoprotein Al precursor (Spot I).

- The expression of these proteins with the course of disease over the period of 120 days was also validated in DEN+2-AAF treated rats sera using Western blot analysis.
- These proteins (three) were further validated in human serum samples through Western blot analysis and showed elevation of their levels in liver cancer patient sera as compared to normal healthy sera.
- cDNA sequence analysis for 17 kDa protein (transthyretin) revealed an open reading frame of 444 bases that resulted in a predicted protein of 147 amino acids.
- The amplified fragments of transthyretin cDNA was directly cloned into pGEM-T Easy vector and the recombinants clones were screened by colony PCR using insert specific primers.
- The sequence analysis of recombinants clones showed 99% homology with the reported nucleotide sequence of tranthyretin and the inversion of the bases were also seen in the cloned sequence. Four bases showed modification.
- The comparison of the amino acids sequence with rat transthyretin sequence showed 98% homology and the changes in three amino acids were found. These substitutions may represent a modified tumor specific protein (transthyretin).
- Expression of transthyretin was achieved using pET-32(a) vector where the 444 bp gene was ligated with Nco I and Bam HI restriction sites. The expression construct pSJ with cloned 444 bp gene of rat liver had a size of 6.3 kb.
- The recombinants were screened, the positive clones were used to transform E. coli expression host strain BL21 (DE3). The cultures were induced with 1mM IPTG for 4 hrs and high level expression of 40 mg/litre culture was achieved in the BL21 (DE3) cells at 1mM IPTG.
- The r-protein was purified on Ni-NTA columns.

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The r-protein was used to check the presence of antibodies in sera of rats bearing liver tumors. Western blot analysis of the r-protein confirmed the presence of anti-transthyretin antibodies in DEN+2-AAF treated rat sera vis-à-vis control sera.

In conclusion, our study suggests the development of liver specific tumors by DEN+2-AAF combination. This model has a number of advantages over the classical models as it does not require partial hepatectomy which involve surgery that causes a lot of pain and mortality of animals. The DEN+2-AAF treatment given to male Wistar rats displayed evidence of oxidative stress and a diminished antioxidant defence system. The impaired oxidant-antioxidant balance represents a risk factor for the development of chronic diseases. The activities of MDA, SOD and CAT were found to be altered in case of treated rats when compared to controls. These parameters might serve as markers for increased oxidative stress in inflammation and various other diseases. Moreover, this animal study has been found to be a suitable model for studying the progression of the disease in animals. The proteomic analysis revealed a number of differentially expressed proteins. The analysis of three of these proteins, namely, complement C3, apolipoprotein AI precursor and transthyretin precursor in diseased state revealed that these proteins get elevated during early stage of cancer development. This observation was subsequently validated in human sera samples also. Our study suggests that these proteins may be prospective early biomarkers for detecting liver cancer at an early stage. Liver cancer cell producing transthyretin protein, its secretion in blood surrounding the local tissue and the production of antibodies against the recombinant protein transthyretin suggests that it may be involved in the biological behavior of this cancer and may serve as a useful marker for cancer cell differentiation, diagnosis and prognosis.