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4.0 RESULTS

Liver cancer remains the leading cause of cancer-related mortality worldwide. Early detection of liver cancer remains difficult due to unavailability of diagnostic markers with high sensitivity and specificity. Proteomics offers a novel approach to study the biological systems by qualitative and quantitative analysis of all the proteins present in the cells and tissues. The present study was designed to analyse and characterize the differently expressed proteins in serum of animals with liver cancer vis-à-vis controls and assess their potential for cancer detection. Liver cancer was induced in male Wistar rats by administration of diethyl nitrosamine (DEN) + 2-acetyl amino fluorine (2-AAF) and the total profile of the serum proteins was analysed by one-dimensional electrophoresis (1DE), two-dimensional electrophoresis (2DE), liquid chromatography-mass spectrometry (LC-MS/MS). The proteins expression levels were validated by western blot analysis and cloning. The results of the study are summarized as follows:

4.1 DEVELOPMENT OF ANIMAL MODEL IN MALE WISTAR RATS BY DEN AND 2-AAF

The rodent model is most widely used animal model as its relatively short life span permits the observation of cellular transformation from its initiation to full-blown malignant cancer. DEN is one of the important environmental carcinogens, found in many commonly consumed foods, and has been reported to induce cancers in animals and humans. Several models have been developed to study multistage carcinogenesis in rat liver; these include the Solt–Farber-resistant hepatocyte model, where the initiation consists of either a necrogenic dose of a hepatocarcinogen or a non-necrogenic dose in conjunction with partial hepatectomy (PH). We have developed a novel protocol for tumor induction in liver which eliminates the need of PH (Malik et al., 2012). Animals were divided into four groups, each group having its own control group. Male Wistar rats were injected with single i.p. dose of DEN (200 mg/kg body weight), controls received saline only and after 1 week of recovery, DEN-treated animals were administered with the repeated doses of 2-AAF (150 mg/kg body weight) orally in 1 % carboxymethyl cellulose that served as promoting agent.
(Figure 4.1). DEN after its metabolic biotransformation produces the promutagenic adducts $O^6$ ethyldeoxy guanosine and $O^4$ and $O^6$ ethyldeoxy thymidine that initiate liver carcinogenesis (James et al., 1985). 2-AAF promotes the growth of DEN altered hepatocytes. The detailed procedure has been described in Materials & Methods, section 3.7.

![Experimental flow chart for liver cancer development in male Wistar rats.](image)

**Figure 4.1** Experimental flow chart for liver cancer development in male Wistar rats.

### 4.2 MORPHOLOGICAL CHANGES

A variety of clinical symptoms were observed in experimental animals after DEN+2-AAF administration; these include blood clotting problems, loss of appetite and water intake. Loss of hair started at day 20 after giving the DEN+2-AAF (Figure 4.2). The amount of food intake was prominently lower, by up to one half, after 90 days of DEN+2-AAF administration. Liver weight increased but body weight decreased with
the disease progression in DEN+2-AAF treated animals significantly over the experimental period as compared to controls. The increase of liver weight might have been caused by small tumors and well developed liver nodules (Table 1). The incidence of preneoplastic foci and enlarged liver were not significant at 30 days in the DEN+2-AAF administrated group, while from 90-120 days the incidence of nodules was present in almost all (100%) the cases in the treated groups. Thereafter, the numbers became greater and their sizes increased. No such changes were observed in the control group (Figure 4.3).

Table 1. Alteration in liver weight and body weight (Control vis-à-vis Treated) during disease progression. Values are expressed as mean ± S.E.M. (n = 12) and followed by student’s Tukey t-test, using GraphPad Prism 5 software, with, P<0.05 as limits of significance.

<table>
<thead>
<tr>
<th>Sr No</th>
<th>Parameter</th>
<th>Groups</th>
<th>0 days</th>
<th>30 days</th>
<th>60 days</th>
<th>90 days</th>
<th>120 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Liver wt (g)</td>
<td>Control</td>
<td>2.95±0.16</td>
<td>3.47±0.22</td>
<td>3.86±0.40</td>
<td>4.49±0.51</td>
<td>5.42±0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>2.93±0.18</td>
<td>3.61±0.61</td>
<td>4.21±0.32</td>
<td>5.11±0.72</td>
<td>6.05±0.67</td>
</tr>
<tr>
<td>2</td>
<td>Body wt (g)</td>
<td>Control</td>
<td>79.5±3.6</td>
<td>109.75±4.2</td>
<td>162±4.8</td>
<td>233.75±5.5</td>
<td>285.5±5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>79.75±3.8</td>
<td>105.75±4.3</td>
<td>151±4.7</td>
<td>221.25±5.1</td>
<td>268±5.8</td>
</tr>
</tbody>
</table>
Figure 4.2 Morphological changes; hair loss started (at day 20) after giving the carcinogen. (A and B - Control, C and D - DEN+2-AAF treated animals).
Figure 4.3 Alteration in livers morphology (Control vis-à-vis Treated) showing disease progression. C - Control, T1 - Treated group (DEN+2-AAF) at 30 days showing normal structure of liver, T2 - Treated group at 60 days showing slightly liver damage, T3 - Treated group at 90 days showing small nodules formation in the liver, T4 - Treated group at 120 days showing large and dense nodules in the liver.
4.3 HISTOPATHOLOGICAL STUDY

It was confirmed by histopathological analysis that liver cancer was induced successfully in rats after administering DEN+2-AAF. The present study on chemically induced hepatocarcinogenesis in rat liver focuses on the basis of induction of neoplastic liver nodules. Neoplastic nodules distort the architecture of premalignant epithelial tissues in both human and experimental animals, much earlier than the onset of cancer. The histopathological examination of the H and E stained control liver sections showed normal architecture of hepatocytes (Figure 4.4 A & B). Liver section of DEN + 2-AAF administered group at 30 days showed vacuolization of hepatocytes in the centrizonal area with variation of nuclear size (Figure 4.4 C & D). A large area of atypical bile duct proliferation was observed at 60 days (Figure 4.4 E & E), while at 90 days details of nodules in the liver parenchyma were prominent. The cells in the nodule were large with clear cytoplasm without sinusoidal spaces (Figure 4.4 G & H). Low power photomicrograph of liver group at 120 days showed clear adenomas in the liver parenchyma. Same section at high power showed the adenomas on the right and normal liver tissue on the left separated by a thin capsule. There was seen clear nuclear atypia in the adenoma (Figure 4.4 I & J). No such defects were observed in the control groups.
Figure 4.4 Photomicrograph showing histopathological changes in liver tissue: (A) Control group at low power (100X) showing normal liver architecture. PT: portal triad; CV: central vein; (B) Same section at high power (400X) showing details of a normal PT; PV: portal vein; BD: bile duct; HA: hepatic artery. (C) Treated group (DEN+2AAF; 30 days) at low power (100X) photomicrograph showing foci of vacuolar change in hepatocytes; (D) Same section at high power power (400X) showing a group of hepatocytes with vacuolated cytoplasm. (E) Treated group
4.4 ANALYSIS OF SERUM BIOCHEMICAL PARAMETERS

Hepatospecific enzymes are activated when hepatocellular damage gives rise to abnormalities in liver function and these enzymes are remarkably increased in HCC. The most sensitive and dramatic indicator of hepatocyte injury is the release of intracellular enzymes, such as transaminases and alkaline phosphatase in circulation after DEN administration. The measurement of alkaline phosphatase (ALP) activity is useful as an indicator of liver function (Jagan et al., 2008). In liver, ALP is closely connected 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. In the present study ALP activities were increased (28.72%, 48.53%, 64.34%, 69.65%) significantly (\( p < 0.05 \)) in treated animals as compared to controls and showed a correlation with the progression of disease from 30 to 120 days respectively (Figure 4.5A).
Aminotransferases (aspartate transaminase and alanine transaminase) are reliable marker enzymes of liver and these are the first enzymes to be used in diagnostic enzymology when liver damage has occurred (Jagan et al., 2008). Because of their intracellular location in the cytosol, toxicity affecting the liver with subsequent breakdown in membrane architecture of the cells leads to their spillage into serum, and their concentration rises in the latter. The activities of aspartate transaminase (AST) and alanine transaminase (ALT) in serum were increased (37.78%, 54.83%, 72.41%, 76.87% and 22.80%, 44.26%, 64.07%, 70.19%) significantly (*P < 0.05) in treated animals in comparison to controls. A correlation was seen between the increase in transaminases activity and the disease progression (Figure 4.5B & C).
Figure 4.5 Serum ALP (A), AST (B) and ALT (C) activities in the control and treated groups. Values are expressed as mean $\pm$ S.E.M. ($n = 12$). Treated group showed a significant increase in ALP, AST and ALT levels as compare to the control group ($^{*}P < 0.05$ treated vs. control group). Values are expressed as mean $\pm$ S.E.M. ($n = 12$).
4.5 ANALYSIS OF HEPATIC OXIDATIVE STRESS PARAMETERS

DEN has been reported to cause an elevated generation of free radicals in the liver, which in turn increases the demand of antioxidant enzymes. Subsequently, it leads to oxidative stress and initiation of carcinogenesis (Janani et al., 2009). Administration of DEN has been reported to generate more LPO products like malondialdehyde (MDA) and 4-hydroxy nonenal. Such findings corroborate with our results as we have seen the elevated level of malondialdehyde in DEN-treated rats (Figure 4.6A). This parameter increased (60.55%, 69.58%, 80.16%, 84.42%) significantly (*p< 0.05) in the treated groups with the progression of the disease from 30 to 120 days respectively.

\[\text{(A)}\]

![Graph showing malondialdehyde levels over time in control and treated groups.](image-url)
Figure 4.6 Activities of malondialdehyde, superoxide dismutase and catalase in control and treated groups. (A) - LPO expressed as nmol MDA formed/min/mg protein. (B) - SOD expressed as nmol epinephrine/mg protein. (C) - CAT expressed as nmol H$_2$O$_2$ consumed/min/mg protein. Values are expressed as mean ± S.E.M. (n = 12).
Chemically induced liver carcinoma is associated with changes in oxygen radical metabolism in liver. The changes in hepatic oxygen radical metabolism were demonstrated by measurement of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT).

SOD is the primary step of the defense mechanism in the antioxidant system against oxidative stress. Tumor cells show a decrease in the activities of SOD and CAT though the mechanism is still unclear. In the present study, the liver SOD, and CAT, activities were decreased (19.18%, 37.24%, 54.23%, 71.50% and 27.93%, 39.84%, 57.97%, 76.60%) significantly (*P < 0.05) in the treated groups with the progression of disease (Figure 4.6B & C), from 30 to 120 days respectively.

4.6 PROTEOMIC ANALYSIS OF RATS SERA

4.6.1 One-dimensional electrophoresis (1-DE)
The 1DE protein expression profiles in sera of DEN+2-AAF treated and control rats were analysed at different time intervals (15, 30, 60, 90 and 120 days). Albumin, the most abundant protein in serum was depleted, and albumin depleted serum was subjected to TCA precipitation. The amount of proteins was estimated by Bradford method. Equal amounts (40 μg) each of protein from different serum samples (representing by different time intervals) was resolved on 15% SDS-polyacrylamide gels. Some of the protein bands (between ~20kDa to ~66kDa range; marked with arrows) in serum samples from treated group were differentially expressed as compared to the control. As shown in the figure 4.7, changes in protein expression started at very early stage (15 days) after giving the carcinogen.
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4.6.2 Two-dimensional electrophoresis (2-DE)

The total serum proteins from control and carcinogen treated animals were subjected to 2D-gel electrophoresis to get better resolution of differentially expressed proteins. The proteins were first separate on pI strips (pH3-10), on the basis of isoelectric point and then on SDS-PAGE on the basis of mass (Details have been given in section 3.15, Materials and Methods). Total of 125 μg proteins each from control and treated serum samples were used for analysis (Figure 4.8).

Figure 4.7 1-D protein profile of control (C) and DEN+2-AAF treated serum samples with disease progression. Changes in protein profiles are marked by arrows. M - Protein molecular weight marker. C - Control, T1 - T5 serum samples collected at 15, 30, 60, 90 and 120 days respectively.
Figure 4.8 2D protein profiles of control and DEN+2-AAF treated serum samples at day 15, acquired using IPG strips (11 cm, pH 3-10).

4.6.3 PD-Quest analysis of 2D- Gels

PD-Quest image analysis software was used to compare computer images of 2D gels to determine differential protein expression. The software landmarked proteins for gel alignment and identified subtle changes in the up or down-regulation of proteins based on intensity of protein staining. PD-Quest software provided quantitative and qualitative information for selecting the best gel(s) for spot cutting from replicate sets of gels. Qualitative differences were considered meaningful when one spot was detected in one gel and not in another. Based on these criteria, it was found that total 46 proteins spots 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). Maximum spots have been found between pH 5-8 (Figure 4.9). An image of the master gel automatically produced by
Figure 4.9 PD-Quest analysis of 2D Gels of control and DEN+2-AAF treated serum samples, acquired using IPG strips (3-10). Spots 1-28 are present only in control while spots 29-32 are present only in DEN+2-AAF treated serum samples. Spots A-N are differentially expressed between control and treated samples.

Table 2. Total protein spots identified by PD-Quest software analysis

<table>
<thead>
<tr>
<th>Protein spots</th>
<th>Expression</th>
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<tr>
<td>14 (A-N)</td>
<td>Differentially expressed between control and treated</td>
</tr>
<tr>
<td>1-28</td>
<td>Present in control</td>
</tr>
<tr>
<td>29-32</td>
<td>Present in treated</td>
</tr>
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</table>

PD-quest software showed all the proteins from control and DEN+2-AAF treated serum samples (Figure 4.10). The red spots are the matched proteins that are quantitatively up-regulated in treated samples versus control, while green are the matched proteins that are quantitatively down-regulated in treated samples versus control, yellow spot on the other hand show qualitative differences between control and treated. On the basis of pixels on gel images, each individual spot was assigned a
quantity to define the differential expression meaningfully. Out of 46 Proteins, four spots G, I, K and N, whose expression level were statistically significant on the basis of expression intensities (P<0.05) were selected for further studies. Spot area and intensity was identified by PD-Quest software and comparison of the four differentially expressed protein spots before and after liver cancer induction revealed increase the intensity of three spots (G, I and N) in treated

Figure 4.10 PD-Quest software generated master gel image by overlaying control and carcinogen treated gels showing all the proteins from control and DEN+2-AAF treated serum samples.
samples over control while the intensity of spot K was decreased in treated serum sample. The quantitative differences in relative concentrations of the proteins are shown in figure 4.11.

4.7 IDENTIFICATION OF PROTEINS BY LC-MS/MS
The four quantitatively expressed protein spots, having maximum difference in their expression levels were characterised by LC-MS/MS. Data analysis was carried out using the database search engine Mascot (www.matrixscience.com). NCBI nr which is a comprehensive, non-identical protein database was used for the sequence search. Redundancy of proteins appearing in the database under different names and accession numbers was eliminated. The taxonomy search was confined to the genus, Rattus, thus ensuring that the hit list only contained entries from the selected species.
4.8 MASCOT SEARCH RESULTS FOR SPOT G

Database : NCBI nr 20070713 (5222402 sequences; 1811015345 residues)
Taxonomy : Rattus (66269 sequences)
Timestamp : 19 Jan 2010 at 08:57:48 GMT
Significant hits : gi/136467 Transthyretin precursor
                   gi/554423 Complement component C3

Figure 4.12 Histogram showing probability based mowse score of transthyretin precursor and complement component C3. Mowse Score is 89 and 87 (>39, P<0.05).
Probability Based Mowse Score

Ions score is $-10 \times \log(P)$, where $P$ is the probability that the observed match is a random event. Individual ions scores > 39 indicate identity or extensive homology ($p<0.05$) and are considered to be the significant scores while scores <39 (in shaded green region) are random hits and not significant. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

MS/MS peptide summary of transthyretin precursor

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<th>Mr(calc)</th>
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<td>471.72</td>
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<td>941.52</td>
<td>-0.09</td>
<td>R.GSPAVDVAVK.V</td>
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<td>42 - 51</td>
<td>471.83</td>
<td>941.65</td>
<td>941.52</td>
<td>0.13</td>
<td>R.GSPAVDVAVK.V</td>
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<tr>
<td>56 - 68</td>
<td>676.80</td>
<td>1351.59</td>
<td>1351.60</td>
<td>-0.02</td>
<td>K.TADGSWEPFASKG.T</td>
</tr>
</tbody>
</table>

Complete peptide sequence: -

1. MASLRLFLLC LAGLIFASEA GPGGAGESKC PLMVKVLDAV RGPVAVDVAV
51. KVFKKTADGS WEPPASGKTA ESGLHGLIT DEKFTEGVYR VELDTKSYWK
101. ALGISPFHEY AEVVFANDS GHRHYTIAAL LSPYSYSTTA VVSNPQN

(Matched peptide sequences are shown in bold red)

MS/MS peptide summary of complement C3

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<td>0.29</td>
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<td>K.ACEPGVDVYVK.T</td>
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</tbody>
</table>

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Complete peptide sequence: -

1  PGSLLRSEET  KQNEGFSLTA  KGKGOGTLTV  VTVYHAKVKG  KTTCKKFDLR
51  VTIKPAPEMA  KKPQDAKSM  ILDICTRYLG  DVDATMSILD  ISMTGIP
101  TNDLELLSSG  VDRYISKYEM  DKAFSNKNTL  IYLKERISHS  EEDCLSF
151  QFFNVGLIQP  GSVKVSYYN  LEESTRFHY  PEKDDGMLSK  LCNMCRCA
201  EENCFMHQSQ  DQVSLNERLD  KACEPGVDYV  YKTCLTTIEL  SDDF
251  IEQVIKSGSD  EQAGQERRF  ISHKCRNAL  K

(Matched peptides are shown in bold red)

4.9. MASCOT SEARCH RESULTS FOR SPOT I

Database : NCBI nr 20120303 (1737829 sequences; 5967463365 residues)

Taxonomy : Rattus (67624 sequences)

Timestamp : 9 Mar 2012 at 06:37:50 GMT

Significant hits: gi|2145145 Apolipoprotein AI precursor

Chromatogram - Apolipoprotein AI precursor

83
Probability Based Mowse Score

Protein score is -10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores > 61 indicate identity or extensive homology (p<0.05) and are considered to be the significant scores while scores <61 (in shaded green region) are random hits and not significant. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

MS/MS peptide summary of apolipoprotein protein AI precursor

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<td>1119.4655</td>
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<td>1194.4204</td>
<td>1194.5305</td>
<td>-92.22</td>
<td>K.WNEEVEAYR.Q</td>
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<td>2128.7763</td>
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<td>2128.0913</td>
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<td>2576.6717</td>
<td>2577.1594</td>
<td>-189.24</td>
<td>K.MQPHLDEFQEKWNEEV</td>
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Complete peptide sequence:

1 MKAAVLAVL VFLTGCQAWE FWQQDEPSQW DRVKDFATVY VDAVKDSGRD
51 **YVSQFE**SSTL GQQLNLNLDD NWDTLGSTVG RLQEQLGPVT QDFWANLEKE
101 TDWPRNEMNK DLENVKQKMQ PHLDEFQEKW** NEEVEAYRQK LEPLATELHK
151 NAKEMQRHLK **VV**E**E**FRDRM RVNADALRAK FG**L**YSQMRE NLAQRLTEIK
201 NHPTLIEYHT KASDHLKTLG EKAP**A**LDDL GQGLMPVLEA WKAKIMSMID
251 EAKKKLNA

(Matched peptides are shown in bold red)

4.10 MASCOT SEARCH RESULTS FOR SPOT K

Database: NCBI 20070713 (5222402 sequences; 1811015345 residues)

Taxonomy: Rattus (66269 sequences)

Timestamp: 9 Jan 2010 at 08:58:43 GMT

Significant hits: gi|121052 Ig gamma-2A chain C region

Chromatogram - IgG-2A chain C region
Figure 4.14 Histogram showing probability based mowse score of Ig gamma-2A chain C region. Mowse Score is 89 (>89, P<0.05).

Probability Based Mowse Score

Ions score is \(-10\times\log(P)\), where P is the probability that the observed match is a random event. Individual ions scores > 40 indicate identity or extensive homology (p<0.05) and are considered to be the significant scores while scores < 40 (in shaded green region) are random hits and not significant. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

MS/MS peptide summary of IgG-2A chain C region

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</table>
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Complete peptide sequence:

1 AETTAPSVP LAPGTALKSN SMVTLGCLVK GYFPEPVTVT WNSGALSSGV
51 HTFPVLQSG LYLTSSVTV PSSTWSSQAV TCNVHAPASS TKVDKKIVPR
101 BCNPCGCTGS EVSSVFIFFP KT KDVLTVTTL TPKVTCLVVD ISQNDPEVRF
151 SWFIDDVEVH TA QTHAPEKQ SNSLRSVSE LP I VH RDLW LN G KTFKCKVNS
201 GAPPAPIEKS ISKPEGTPRG PQVYTMAPPK EEMTQSQVSI TCMVKGFPY
251 DIYTEWKMNQ QPQENYKNTP PTMTDGSYF LYSKLNVKKE TWQQGNTFTC
301 SVLHEGLHNN HTEKLSSHSP GK

(Matched peptides are shown in bold red)

4.11 MASCOT SEARCH RESULTS FOR SPOT N

Database : NCBI nr 20070713 (5222402 sequences; 1811015345 residues)
Taxonomy : Rattus (66269 sequences)
Timestamp : 19 Jan 2010 at 09:00:31 GMT
Significant hits: gi|62660301 Immunoglobulin J chain isoform 2

Chromatogram - Immunoglobulin J chain isoform 2
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Figure 4.15 Histogram showing probability based mouse score of immunoglobulin J chain isoform 2. Mouse Score is 106 (>40, P<0.05).

Probability Based Mouse Score
Ions score is -10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores > 40 indicate identity or extensive homology (p<0.05) and are considered to be the significant scores while scores <40 (in shaded green region) are random hits and not significant.

MS/MS peptide summary of immunoglobulin J chain isoform 2

<table>
<thead>
<tr>
<th>Start - End</th>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>Delta</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>43 - 57</td>
<td>575.03</td>
<td>1722.07</td>
<td>1721.85</td>
<td>0.22</td>
<td>R.IIPSPEDPNEDIVER.S</td>
</tr>
<tr>
<td>61 - 68</td>
<td>462.84</td>
<td>923.67</td>
<td>923.56</td>
<td>0.11</td>
<td>R.IVVPPLNNR.E</td>
</tr>
<tr>
<td>61 - 68</td>
<td>462.91</td>
<td>923.81</td>
<td>923.56</td>
<td>0.25</td>
<td>R.IVVPPLNNR.E</td>
</tr>
<tr>
<td>146 - 159</td>
<td>799.47</td>
<td>1596.93</td>
<td>1596.68</td>
<td>0.25</td>
<td>K.MVQTALTPDSCYPD</td>
</tr>
</tbody>
</table>
Complete peptide sequence:

1 MKTHLLLWGV LAIFVKAVIV TGDNKGTILA DNKCMCTRIT SRIIPSPEDP
51 NEDIVERSIR IVVPLNNREN ISDPTSPVRT NFVYHLSDVC KKCDPVEVEL
101 EDQVVTATQS NICSGDSGVP ETCYMYDRNK CYTAMVPLRY HGETKMVQTA
151 LTPDSCYPD

(Matched peptides are shown in bold red)

The details of the Sequence coverage (%), molecular weights and pIs of each of these proteins have been summarized in Table 3.

Table 3. Quantitatively expressed proteins identified by LC-MS/MS in liver cancer induced rats sera at 15 days

<table>
<thead>
<tr>
<th>Spots</th>
<th>NCBI accession No</th>
<th>Protein Name</th>
<th>Sequence coverage (%)</th>
<th>Protein MW (kDa)</th>
<th>pI</th>
<th>Mowse Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>gi</td>
<td>136467</td>
<td>Tranthyretin precursor</td>
<td>15</td>
<td>15.824</td>
<td>5.77</td>
</tr>
<tr>
<td></td>
<td>gi</td>
<td>551423</td>
<td>Complement component C3)</td>
<td>7</td>
<td>32.414</td>
<td>5.73</td>
</tr>
<tr>
<td>I</td>
<td>gi</td>
<td>2145145</td>
<td>Apolipoprotein AI precursor</td>
<td>41.5</td>
<td>29.956</td>
<td>5.45</td>
</tr>
<tr>
<td>K</td>
<td>gi</td>
<td>121052</td>
<td>Immunoglobulin gamma 2A chain C region</td>
<td>9</td>
<td>35.677</td>
<td>7.72</td>
</tr>
<tr>
<td>N</td>
<td>gi</td>
<td>62660301</td>
<td>Immunoglobulin J chain isoform 2</td>
<td>23</td>
<td>18.229</td>
<td>5.18</td>
</tr>
</tbody>
</table>

4.12 PROTEINS VALIDATION BY WESTERN BLOT ANALYSIS IN RATS SERA

Western blots with protein specific antibodies were performed to validate the presence of each of the three proteins (namely, complement C3, transthyretin and apolipoprotein AI precursor) and studied the changes in their expression levels in sera of DEN+2-AAF treated rats with the progression of liver cancer. Relative changes in the expression of a specific protein are measured on western blots by forming the ratio of the densitometric values of bands containing the protein between control and DEN+2-AAF treated samples.
Complement components are important mediators of inflammation and contribute to the regulation of the immune response. In cancer patients, complement activation with subsequent deposition of complement components on tumor tissue has been demonstrated (Neng et al., 2006). Western blot analysis for Complement C3 showed that substantial increase in the complement C3 expression levels at different time interval (15, 30, 60, 90 and 120 days post treatment respectively; Lanes 2-6, Figure 4.16A). The quantitation of western blot showed complement C3 protein up-regulation in treated sera as compared to control and remains almost same with the progression of disease. This analysis validated the complement C3 expression levels in the sera of animals with liver cancer vis-à-vis controls (Figure 4.16 B). The elevation in levels of C3 takes place in early phase (15 days or earlier) of the disease and the levels remain elevated throughout the study period without any further increase in its concentration.

**Figure 4.16 (A)** Western blot analysis of complement C3 confirms the up-regulation of the protein in DEN+2-AAF treated rats' sera in comparison to control sera. (B) Bar showing intensity of C3 and each protein band was quantified using Image J software analysis. C - Control, Lane T1 - T5 DEN+2-AAF treated sera.
Transthyretin (Prealbumin) is a plasma protein involved in the transport of retinol and thyroxine. The liver is the main site of transthyretin synthesis in rats as well as humans. 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 (Beate et al., 2005). In the present study, we observed the up-regulation of this protein in DEN+2-AAF treated rats sera. The expression levels of transthyretin precursor protein were increased at different time interval (15, 30, 60, 90 and 120 days post treatment respectively; Lanes 2-6) in treated rats sera samples in comparison to control. The levels almost peaked on day 30 and remained elevated during the disease progression (Figure 4.17A).

![Western blot analysis of transthyretin confirms the up-regulation of the protein in DEN+2-AAF treated rats sera in comparison to control sera.](image1)

![Bar showing intensity of Transthyretin and each protein band was quantified using Image J software analysis. C - Control, Lane T1 - T5 DEN+2-AAF treated sera.](image2)

**Figure 4.17** (A) Western blot analysis of transthyretin confirms the up-regulation of the protein in DEN+2-AAF treated rats sera in comparison to control sera. (B) Bar showing intensity of Transthyretin and each protein band was quantified using Image J software analysis. C - Control, Lane T1 - T5 DEN+2-AAF treated sera.
Liver is the main organ for the synthesis, storage, transportation and degradation of some apolipoproteins. The proportion of proapoAI showed a tendency toward increase under advanced liver damage because liver participates in the process of converting proapo AI to the mature apoAI (Isobe et al., 1990). We found the elevated expression of apolipoprotein AI precursor (proapoAI) reached to maximum at day 30 and remained elevated at different time interval (15, 30, 60, 90 and 120 days post treatment respectively; Lanes 2-6) in treated rats sera samples in comparison to control (Fig 4.18A).

**Figure 4.18** (A) Western blot analysis of apolipoprotein AI confirms the up-regulation of the protein in DEN+2-AAF treated rats sera in comparison to control sera. (B) Bar showing intensity of apolipoprotein AI and each protein band was quantified using Image J software. C - Control, Lane T1 - T5 DEN+2-AAF treated sera.
The quantitation of transthyretin precursor and apolipoprotein AI precursor showed the up-regulation of these proteins in treated sera as compared to control and remains almost same with the progression of disease (Figure 4.17B & 4.18B). The above results confirmed the 2-DE and LC-MS/MS results in chemically induced liver cancer animals.

**4.13 PROTEINS VALIDATION BY WESTERN BLOT ANALYSIS IN HUMAN SERA**

To validate the presence and the expression levels of these 3 proteins complement component C3, transthyretin precursor (TTR) and apolipoprotein AI precursor (preapoAI), western blot analysis was carried out in human sera from healthy human and liver cancer patient (clinically confirmed cases of liver cancer). As shown in figure 4.19 A, B and C, all the three proteins showed elevation of their levels in liver cancer patient sera as compared to normal healthy sera.

**Figure 4.19** Western blot analysis with the specific C3 (A), TTR (B) and ApoAI precursor (C) antibodies confirms the up-regulation of these proteins in human sera samples in comparison to control. Bands were quantified by Image J software (C-Control human sera; T- Liver cancer patient sera).
The data of animal studies and analysis of human samples taken together show that these proteins have a potential for the development of biomarkers for early detection of HCC.

4.14 cDNA SYNTHESIS, CLONING AND EXPRESSION

4.14.1 Analysis of total RNA from liver tissue
Total cellular RNA was isolated from liver of rats by Tri reagent. 100 mg of each tissue sample was homogenized in 1 ml of Tri reagent. Finally the RNA pellet was dissolved in DEPC treated sterile water. Spectrophotometric measurements of RNA samples at 260 nm and 280 nm gave an absorbance ratio (A 260/A 280) of 1.8 indicating high purity. Quality of RNA was checked on 1.2% formaldehyde gel. Two clear bands of ribosomal RNA (28S and 18S) were visible together, showing that the RNA isolated is pure and intact (without any degradation) as shown in result (Figure 4.20). RNA was used to make cDNA and amplified using inserts specific primers by semi quantitative RT-PCR.

![Figure 4.20](image)

Figure 4.20 Qualitative analysis of total RNA isolated from liver tissue of rat through agarose gel electrophoresis. Lane 1 - Control, lane 2 - Treated.

4.15 NUCLEOTIDE SEQUENCE ANALYSIS
Based on NCBI-BLAST complementation analysis, the amino acid sequences of the protein (Transthyretin precursor) was used to find the sequence of gene from the gene
Restriction analysis of the gene was performed by submitting the gene sequence to NEB cutter V2.0 DNA programme. Sequence specific forward and reverse primers were synthesized. The \textit{Nco I} and \textit{Bam HI} restriction sites were included at the 5' end of the forward and reverse primer respectively. The primers were used to amplify the cDNA by RT-PCR. The sequence of the primers used was:

\textbf{Forward primer:} \textit{5'} CATGCCATGGGATTTCCCTTCGCTGTTC \textit{3'}

\textbf{Reverse primer:} \textit{5'} GATCGGATCCCTCATGTTCTGGGGGTATTACTGAC \textit{3’}

\begin{verbatim}
ATGGCTTCCCTTCGCTGGAGATATTTTGCTGGTCTAAGGCT
GGCCCTGGGGGTCTGGAGAATCCAAGTGCTCCTGCTGATGCTACTGCTGTC
CGAGGACGCCGCTCTGCTGCTGCTGGGAGATATTTTGCTGGTCTAAGGCT
TGAGGACGCCGCTCTGCTGCTGCTGGGAGATATTTTGCTGGTCTAAGGCT
GATGAGAAGATTCAGGGGTTACAGGATAGACACCGAGATGCTACTGCTGTC
GCTCTGGCATTTCTGTTCCATATCCAAGTGCTCCTGCTGCTGCTGTC
GGTCATGCTCCACTACACCCTCAGCAGGCTGCTGCTGCTGCTGTC
GTGTCAGTAACCCAGAATCTGAC
\end{verbatim}

\textbf{Figure 4.21} Nucleotide sequence of transthyretin precursor (\textit{Rattus norvegicus}).

\subsection{4.16 cDNA Amplification by RT-PCR}

The cDNA for transthyretin precursor was synthesized by RT-PCR using rat liver RNA as template. Three \( \mu \)g of total RNA was used for each 20 \( \mu \)l of reverse transcriptase reaction. One \( \mu \)l of cDNA product was further used for PCR amplification in a 25 \( \mu \)l reaction using gene specific forward and reverse primers. The amplified products were separated on a 1\% agarose gel, visualized by ethidium bromide (0.5\( \mu \)g/ml) staining. A single band of amplified cDNA was distinctly visible corresponding to 444bp as expected for the transthyretin gene (Figure 4.22).
The amplified fragment was eluted, purified and visualized on 1% agarose gel (Figure 4.23).
4.17 MOLECULAR CLONING OF GENE IN PGEM-T EASY VECTOR

The cDNA was cloned in pGEM-T Easy vector by TA cloning which is a simple and an efficient method for cloning of PCR products. The procedure exploits the terminal transferase activity of certain thermophilic polymerases including Taq DNA polymerase that adds a single adenosine residue to the 3' ends of the double stranded molecule. Therefore, most of the molecules amplified by Taq DNA polymerase possess single 3'-A overhang. The use of a linearised T vector with 3'–T overhangs on both ends allows direct high efficiency cloning of PCR products carrying this 3'–A overhang. The map of the T vector is shown in figure 4.24. The vector carries ampicillin resistance selectable marker. The MCS is within the lac Z α fragment that allows blue-white screening. The insert can be released using Nco I enzyme or by double digestion. The cloning region is flanked by SP6 and T7 RNA polymerase promoter sequences. The molar ratio of vector DNA to insert was kept 1:3. Ligation reaction was set up as described in Materials and Methods. The ligation reaction was terminated by heat inactivation and the ligation mixture was used for transformation of E.coli DH-5α cells. The transformed cells were plated on LB - agar ampicillin plates containing IPTG and X-gal. After an overnight incubation at 37°C, blue and white (recombinants) colonies became visible on the plates (Figure 4.25).

Figure 4.24 Map of T vector
4.18 SCREENING OF RECOMBINANT CLONES BY COLONY PCR

A number of colonies were picked randomly from the ampicillin containing LB-agar plates. The clones were further identified by colony PCR amplification screening using insert specific forward and reverse primers. The positive clones gave a strong amplification of desired lengths (Figure 4.26).

**Figure 4.25** LB agar plate showing recombinants clones (white colony), and non-recombinant (blue colony).

**Figure 4.26** Colony PCR of the r-clones grown on ampicillin containing LB agar plates. Lane 1 - 100 bp plus DNA ladder, Lanes 4, 6 and 7 showing strong amplification of r-clones.
4.19 CONFIRMATION OF R-CLONES BY RE-DIGESTION

Plasmid DNA was isolated by alkaline lysis method and analyzed for the presence of insert by restriction analysis as procedure described in Materials and Methods. Double restriction digestion using Nco I and Bam HI enzymes was done to pop out the 444 bp insert (Figure 4.27).

![Figure 4.27](image)

**Figure 4.27** Restriction digestion of r-plasmids and 444 bp insert was released. Lanes 1-3 showing RE-digestion of r-clones. Lane M - 1 kb DNA ladder.

4.20 SEQUENCE ANALYSIS OF THE CLONE

The recombinant clone was sequenced by commercially employed automated DNA sequencer (ABI 3130 Genetic Analyzer; Chromas Biotech India Pvt. Ltd. Bangalore, India). The sequence was reconfirmed and it showed 99% homology with the reported nucleotide sequence of tranthyretin. The transversion of the bases were also seen in the cloned sequence (Figure 4.28).

![Sequence](image)

**Figure 4.28** Sequence of PCR positive clone using forward primer. Highlighted region is the restriction enzyme site. Initiation and stop codon are underlined.
The alignment and comparison of the sequence with reported sequence of the protein is shown in figure 4.29.

**Figure 4.29** The sequence alignment of gene showing homology with transthyretin by BLAST analysis. Highlighted region showing transversion of bases.

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).
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<table>
<thead>
<tr>
<th>GENE ID: 24856 Ttr [Rattus norvegicus]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score = 266 bits (680), Expect = 7e-89,</td>
</tr>
<tr>
<td>Identities = 144/147 (98%), Positives = 144/147 (98%), Gaps = 0/147 (0%)</td>
</tr>
<tr>
<td>Frame = +1</td>
</tr>
</tbody>
</table>

Query 1  MASLRLFLLCLAGLIFASEAGPGGAGESKCPLMVKLDAVRGSPA VDVAVKVF KKTADGS
Sbjct 1  MASLRLFLLCLAGLIFASEAGPGGAGESKCPLMVKLDAVRGSPA VDVAVKVF KKTADGS

Query 181 REPFASGKT AESGELHGLTTDEKFTEGV YRVELDT SYWNALG ISPHEYA E V V TANDS
Sbjct 61 WEPFASGKT AESGELHGLTTDEKFTEGV YRVELDT SYWKalG ISPHEYA E V V TANDS

Query 361 GHRHYTIAALLSPYSYSTTAWSNPQN
Sbjct 121 GHRHYTIAALLSPYSYSTTAWSNPQN 147

Figure 4.30 Amino acids sequence of transthyretin protein deduced from translated nucleotide, arrow showing modification of amino acid.

The change in three amino acids 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 (see later).

4.2.1 SUB CLONING OF GENE IN VECTOR PET-32(A)

A construct for expression the transthyretin in Ecoli was made in the pET-32(a+) vector (Novagen) to express the gene product as a His-tagged fusion protein. The vector permits production of protein insoluble form and facilitates rapid purification by affinity chromatography on Ni-NTA column. The map of pET-32(a) is shown in figure 4.31 and the features of vector are summarized in the Table 4. The amplified fragment was eluted, purified, digested with Neo I/Bam HI and sub cloned into pET-32(a) expression vector, yielding the expression construct pSJ-6.3 kb (Figure 4.32). In the construct pSJ-6.3, the inserted gene is fused in frame with ATG codon (at 5’end) and 6x His tag coding sequence (at 3’end) present in the vector. Ligation reaction was set up as described in Materials and Methods and the ligation mixture was used for transformation of E.coli BL21 (DE3) cells. The transformants were selected by plating the cells on LB-agar plates containing ampicillin. After an
overnight incubation at 37°C, white colonies became visible on the plates (Figure 4.33).

Figure 4.31 Map of pET-32(a) vector
## Table 4 Components and features of pET32 (a) vector

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. T7 promotor</td>
<td>T7 promoter which is specific to only T7 RNA polymerase (not bacterial RNA polymerase) and also does not occur anywhere in the prokaryotic genome</td>
</tr>
<tr>
<td>2. lac operator</td>
<td>which can block transcription</td>
</tr>
<tr>
<td>3. His-tag coding sequence</td>
<td>3’ to the polylinker cloning region</td>
</tr>
<tr>
<td>3. Translation stop codons</td>
<td>In all reading frames for convenient preparation of expression constructs</td>
</tr>
<tr>
<td>5. Synthetic ribosomal binding site</td>
<td>For efficient translation</td>
</tr>
<tr>
<td>6. LacI gene</td>
<td>This codes for the lac repressor protein</td>
</tr>
<tr>
<td>7. Ap</td>
<td>Ampicillin resistance marker</td>
</tr>
<tr>
<td>8. pBR 322</td>
<td>Plasmid origin of replication</td>
</tr>
<tr>
<td>9. f 1</td>
<td>Single-stranded DNA synthesis</td>
</tr>
</tbody>
</table>

### Figure 4.32
Map of Expression construct with 444 bp fused gene. Expression construct was created by inserting 444 bp gene in pET - 32(a) vector. The size of the clone pSJ was 6.3 kb.
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4.22 SCREENING OF RECOMBINANT CLONES BY COLONY PCR
A number of white colonies were picked randomly from the ampicillin containing LB-agar plates and confirmation of recombinant clones was done by colony PCR using insert specific forward and reverse primers. The positive clones showed the strong amplification of the insert band of 444 bp (Figure 4.34). Further confirmation of r-clones was done by double restriction digestion (NcoI/Bam HI) of the plasmid DNA to release insert (Figure 4.35).

Figure 4.33 LB agar plate with the transformed colonies of E. coli BL21

Figure 4.34 Colony PCR of the r-clones grown on ampicillin containing LB agar plates. Lanes 1, 3 and 6 showing strong amplification r-clones. Lane 7 - 100 bp plus DNA ladder.
4.23 EXPRESSION OF 17 KDA PROTEIN IN E. COLI BL21

Small scale expression of clones was carried out as described in Materials and Methods section to check for the expression of 17 kDa protein. Several recombinant clones were inoculated in 5ml cultures, and 1 ml of an overnight culture was used to inoculate 250 ml of fresh medium. The cells were grown at 37°C until the OD$_{600}$ reached to 0.8 and 1mM IPTG was added to the medium. Uninduced cultures were kept separately as control. Total lysates (prepared by directly boiling the cell pellets in SDS-PAGE sample buffer) were examined. The expression results are shown in figure 4.36. It is evident from the comparison of the polypeptide profiles obtained in the absence (lane 3) and presence (lane 4) of IPTG that induction of 444 bp gene expression results in the appearance of a new ~17 kDa band, which is consistent with the predicted size of expressed protein. The analysis showed the r-protein was expressed as inclusion bodies.
4.24 PURIFICATION OF INDUCED PROTEIN BY NI-NTA AFFINITY CHROMATOGRAPHY

The strategy for isolation of the induced protein is shown in figure 4.37. Ni-NTA affinity chromatography was performed under denaturing conditions to purify the protein. The high affinity of the Ni-NTA resins for 6xHis-tagged proteins or peptides is due to the strength with which these ions are held to the NTA resin. NTA has a tetradeutate chelating group that occupies four of six sites in the nickel coordination sphere. A denaturing buffer containing 8M urea was used to solubilize inclusion bodies containing 6xHis-tagged proteins. The fractions collected during different steps of the purification were analyzed by SDS-PAGE as shown in Figure 4.38. Elution with a pH 4.5 buffer resulted in the emergence of highly purified recombinant protein from the column (lanes 2 to 6).
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Figure 4.37 Strategy for purifying His tag fused protein on Ni-NTA column.
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Figure 4.38 Single step purification of recombinant 6x-His tag expressed 17 kDa protein using Ni-NTA. Lane 1 - prestain protein marker, Lanes 2-6 protein elution fractions (flow through) collected in buffer 3 (pH-4.5).

4.25 QUANTITATIVE ESTIMATION OF PURIFIED PROTEIN
Transthyretin protein purified by Ni-NTA affinity column was quantified by Bradford method. The yield of r.protein was found to be ~40 mg/l of culture.

4.26 IMMUNOBLOT ANALYSIS AND VALIDATION OF ANTIBODY RESPONSE AGAINST THE RECOMBINANT PROTEIN IN RAT SERA
As over expression of a protein or modification of a normal protein during tumorogenesis making it tumor specific may evoke immune response, it was interesting to see if antibodies to it would be present in serum of disease rats. In order to check the presence of antibodies against transthyretin, immunoblot analysis was carried out using rat sera of control as well as treated groups (15, 30, 60, 90 and 120 days respectively) as primary antibody. For immunoblot analysis, purified recombinant protein was resolved on SDS-PAGE and transferred on nitrocellulose membrane at 100 V, 4°C for 2-3 hr. The blot was then blocked with solution containing 3% BSA and 0.1 Tween 20 in Tris-buffer saline (TBS) for 1 hr at RT. The
membrane containing the bound protein was incubated with treated rats sera (1:50) as well as control sera. Recombinant-protein reacted with treated sera only and showing a single band of 17 kDa. It was confirmed that the antibodies for transthyretin protein were present in treated sera. The antibodies response progressively increased in treated rats sera at 30-120 days respectively. It was observed that the immune response was evident at 30 days in treated group, much earlier than tumor formation in them. No antibody response was observed in the control (Figure 4.39).

Figure 4.39 Immunoblot analysis of the recombinant protein transthyretin detected with rat sera, confirming the presence of anti- transthyretin antibodies in serum samples of DEN+2-AAF treated group in comparison to control. Control group showed no antibody response. Lane 1 - prestain protein marker, lane 2 - control and lanes 3 - 7 treated groups at different time interval (15, 30, 60, 90 and 120 days respectively).