

Identification Of Liver Mitochondria Tumor Associated Antigens Of The Mice Exposed To N, Nitrosodibutylamine: A Promising Strategy In Development Of Anticancer Treatment

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Abstract

Background: N-Nitrosodibutylamine possess the ability to induce bladder tumor as well as in the liver, and oesophagus when it is administered in the body. Exposure to N-Nitrosodibutylamine can happen by different modes such as by ingestion, inhalation and dermal contact.

Methods: In the present investigation an attempt has been done to identify, isolate as well to purify the tumor associated antigens (TAA) from the liver mitochondria of the mice when exposed to N-Nitrosodibutylamine.

Results: It was found that mitochondrial membrane protein of N-Nitrosodibutylamine-exposed animals exhibited differential expression of protein when compared with the control animals. A low molecular weight (~14 kDa) protein was found to be over expressed significantly on liver mitochondrial membrane upon N-Nitrosodibutylamine exposure in mice as compared with the normal control and identified as TAA, showing the sign that some of the proteins could be used as TAA for further study.

Conclusion: These identification of TAAs will provide the basis for the use of mitochondria as markers for the early detection for the treatment of liver cancer.

Keywords: TAA, Liver cancer, liver mitochondria, N-Nitrosodibutylamine.

INTRODUCTION

It is nearly impossible to prove what causes cancer in any individual because most cancers have multiple possible causes. The great majority of cancers, some 90–95% of cases, are due to environmental factors. The remaining 5–10% are due to inherited genetics (Anand et al., 2008). Environmental factors as used by cancer researchers indicates any cause that is not inherited genetically, such as lifestyle, economic and behavioural factors, and not merely pollution (Kravchenko et al., 2009).

N-Nitrosodibutylamine is a class of reported to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals and has been found to have a half-life of 2.8 days (Matsushiet al., 1996). N-Nitrosodibutylamine has been reported to exhibit the ability to induce tumors in bladder, and oesophagus on its administration in the body. It causes cancer by different modes of exposure, such as ingestion, inhalation, and dermal contact such as dermal contact.

The strongly carcinogenic activity of nitrosamines, which for 90% of all animals tested is set out in N-nitroso compounds, is a consequence of the metabolic formation of the reactive carbenium ion, a strong alkylating reagent. This electrophilic ion reacts with the electron-rich atoms of DNA -base. Alkylation takes place mainly at the O6 and N7 positions of guanine (John A., et al. 2009) (Bradfield, 2009). and can lead to several effects.

Mitochondria has not only been reported to play an important role in several disease but its importance also plays an important role in respiratory chain, as defects in additional mitochondrial functions and behaviours have been found to be associated to cancer, metabolic disorders, and neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's disease (Nunnari et al., 2012).

As mitochondria plays a dominant role in controlling various parameters so changes in these parameters can affect the biosynthetic pathways, cellular signal transduction pathways, transcription factors and chromatin structure to shift the cell from a naive, differentiated state to an actively proliferating one. Analysis of the consequences of mitochondrial gene mutations is providing broad new insights into the importance and complexity of mitochondrial alterations in cancer, though mutations in the mitochondrial DNA in cancer cells have been recognized for more than two decades (Horton et al., 1996). Role of mitochondrial alterations in cancer came to attention and interest when mitochondrial tricarboxylic acid (TCA) cycle gene mutations in cancer cells was discovered.

Main goal of treatment is complete removal of cancer without any damage to the rest of the body.

One of the modest approaches to improve systemic cancer therapy is the specific active immunotherapy with preparations containing purified TAA instead of the whole tumor cell (Mine et al., 1999). The TAA which is present mainly in the tumor cells are growth factors, growth factors receptors as well as oncogene-encoded protein. Oncofetal tumor antigens are found not only in the cancerous cell but also seen on normal fetal cells (Urban et al., 1992). The increased level of these can be recognized by the immune system, which mounts an immune response and destroys these cells without damaging any normal cells (Kuby, 1997). Isolation and immunochemical characterization of certain TAA enable the study of immune reactions against highly purified TAA with encouraging results in cancer patients.

Targeting the liver mitochondria TAA of the mice exposed to N-Nitrosodibutylamine in the present study appears to be a promising strategy for the search of novel anticancer treatment as well as the drugs. The potential of this field may be utilized in the identification of new markers and risk assessment as well as therapeutic targets.

MATERIALS AND METHODS EXPERIMENTAL ANIMALS

Swiss albino mice (BALB/c) 10 numbers in each group, bred by random breeding at the animal house of the department were kept on basal diet ad libitum in plastic cages at the temperature-controlled animal room ($21 \pm 2^\circ\text{C}$),

at 12 hours light and dark cycle. At the start of the experiment, the mice were 6-8 weeks old weighing around 22-25 gm in weight. Sex chosen for the experiment was female.

CANCER INDUCTION

A weekly dose of 10 mg per kg body weight of N-Nitrosodibutylamine in 5% ethanol was administered intravenously in healthy female mice of 6-8 weeks old weighing around 22-25 gm for a period of 16 weeks and sacrificed at the end of treatment as required. Age-matched sham-treated mice served as control. All animal procedures were performed according to approved protocol and in accordance with recommendations for the proper use and the care of the laboratory animals. The induction of carcinogenesis was followed by monitoring various parameters such as GGT, AChE, GST, SGOT, and SGPT. Beside this histological examination was also done (Dutta et al., 2006).

MITOCHONDRIAL MEMBRANE PROTEIN ISOLATION PROTEIN EXTRACTION

The mitochondrial membrane protein was isolated from the liver mitochondria of the normal control mice and treated mice by method described by Frilabo, with slight modifications.

Requirements

- 25mM HEPES-KOH (pH 7.6 adjust with KOH)

- 1mMDTT
- 1MM PMSF
- 10%Glycerol
- 0.5mMEDTA
- 5mMMgCl₂
- 0.5 M KCl
- Lysis buffer: To make 250 ml of lysis buffer the following are added:25mM HEPES-KOH,1mM DTT,1mM PMSF,10% glycerol,0.5mM EDTA and5mM MgCl₂ and mixed well.
- 0.5% Tween20

Procedure

In brief,the isolated mitochondria were re-suspended in 1/3 the packed cell volume lysis buffer (25mMHEPES-KOH pH 7.6; 5 mM MgCl₂; 0.5 mM EDTA; 10% Glycerol; 1 mM DTT; 1mM PMSF). The suspension was put into a glass homogenizer and homogenize with a tight pestle. 0.5% Tween 20 and 1M KCl were then added. The mixture was incubated on ice for 5 minutes. The homogenization is repeated 10 times and the final mitochondrial lysate was spun at 100,000 g in an ultracentrifuge (Thermo Scientific Sorvall EX Ultra 100 centrifuge) at 4°C for 60 minutes. The clear supernatant was carefully collected, avoiding the fluffy layer over the pellet, to yield the final protein fraction. The protein fractions were then stored in aliquots and stored at -80°C.

IDENTIFICATION AND PURIFICATION OF THE TAA SDS-PAGE ANALYSIS OF THE EXTRACT

The mitochondrial protein extracts obtained from the control and treated mice were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis to resolve the proteins and identify the overexpressed proteins as TAA of our interest.

Requirements

- Acrylamide stock solution:29.2gm of acrylamide and 0.8gm of bisacrylamide were dissolved in a final volume of 100ml in distilled water.
- 1.5 M Tris-HCl buffer(pH 8.8)
- 0.5M Tris-HCl buffer(pH6.8)
- 10% Ammonium persulphate
- 10% Sodium dodecyl sulphate
- 1X Electrode reservoir buffer: 0.025 M Tris,0.19 M Glycine and 0.1% SDS adjusted to a pH of 8.3.
- 5X sample buffer: 5ml of 0.5 M Tris-HCl buffer pH 6.8,5 gm sucrose,0.25 ml mercapthoethanol,1ml of 0.5% bromophenol blue made up to a final volume of 10 ml with double distilled water.
- TEMED

Procedure

Acrylamide discontinuous gels (resolving gel 15% and stacking gel 4%) were prepared according to the procedure described by (Laemmli, 1970). Briefly, a mixture of 2.35ml of distilled water, 2.5ml of 1.5M Tris-HCl pH 8.8, 5ml of acrylamide, 100µl of 10% SDS, 5µl of TEMED and 50µl of 10% APS was used for making the resolving gel. The solution was mixed gently and carefully poured between the glass plates assembled for the electrophoretic run and was left for polymerization. A space of 3cm was left on the top of the gel for the casting of stacking gel. The stacking gel of 4% was made by mixing 6.1ml of distilled water, 2.5ml of 0.5M Tris-HCl pH 6.8, 1.33 ml of acrylamide, 100µl of 10% SDS, 10µl of TEMED and 50µl of 10% APS and poured over the polymerized resolving gel and the comb was inserted into it to make wells for loading the samples. The comb was removed after polymerization of the gel to which protein sample is to be loaded. The whole setup was then transferred to the electrophoretic chamber, and reservoir buffer solution was poured slowly. 25µl of the protein sample was mixed with 6.25µl of sample buffer and heated in boiling water for about 3 mins. The sample was then loaded into wells. Molecular weight markers were also loaded onto the gel. The gel was run at a constant voltage of 200V and a current of 60mA for 45mins. After completion of run gel was fixed in methanol/acetic acid, stained with coomassie brilliant blue R250 till protein bands are observed and destained with methanol/acetic acid for a few minutes.

ION-EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography was employed in order to separate and identify the protein.

Ion-exchange Chromatography is a widely used technique for the separation of proteins on the basis of Coulombic interaction. It has two types:

- Cation: for separating the positively charged proteins
- Anion: for separating negatively charged proteins.

In order to separate and purify the TAA, the crude mitochondrial protein extract was subjected to cation exchange chromatography.

Cation-exchange chromatography: The crude extract from both the normal and treated were run individually on CM-Sephadex C50.

Requirements

- CM-Sephadex C50.
- 50mM Phosphate buffer, pH 7: Buffer A.
- 100mM NaCl in buffer A: Buffer B.

Procedure

The concentration of the protein in the mitochondrial protein extract was estimated by the Bradford's method (Bradford M.M., 1976). It was then dialyzed overnight against buffer A at room temperature. The column was packed with the CM-Sephadex C50 matrix and was washed with buffer A. Unbound proteins were eluted out with the same buffer A. The proteins bound to exchanger were then eluted out from the column by applying a linear gradient of approximately 100mM NaCl Buffer B. Fractions of 1.5 ml were collected and the absorbance were read at 280nm.

PURIFICATION OF TAA FROM SDS-PAGE GEL

The unbound protein obtained from the cation-exchange chromatography column was subjected to preparative SDS-PAGE for the further purification.

Requirements

- Acrylamide stock solution: 29.2 gm of acrylamide and 0.8 gm of bis-acrylamide were dissolved in a final volume of 100 ml in distilled water.
- 1.5 M Tris-HCl buffer (pH 8.8)
- 0.5 M Tris-HCl buffer (pH 6.8)
- 10% Ammonium persulphate
- 10% Sodium dodecyl sulphate
- 1X Electrode reservoir buffer: 0.025 M Tris, 0.19 M Glycine and 0.1% SDS adjusted to a pH of 8.3.
- 5X sample buffer: 5 ml of 0.5 M Tris-HCl buffer pH 6.8, 5 gm sucrose, 0.25 ml mercaptoethanol, 1 ml of 0.5% bromophenol blue made up to a final volume of 10 ml with double distilled water.
- TEMED

Procedure

The fractions corresponding to peaks obtained from the cation exchange chromatography column were run on SDS-PAGE along with a crude extract. A longitudinal section of the gel corresponding to the crude extract lane was cut and stained. This was used as a reference to locate the exact position of the TAA in the rest of the gel. The portion of the gel corresponding to the TAA was cut off the unstained gel. It was cut into small pieces and homogenized in 3% 1-butanol. The homogenate was centrifuged at 8000g for 30 min at 4°C. The supernatant was collected and the pellet was resuspended in 3% 1-butanol and centrifuged. The supernatant collected was pooled, dialyzed against distilled water overnight and lyophilized. It was then run on an SDS-PAGE gel.

MOLECULAR WEIGHT DETERMINATION OF TAA

Gel filtration chromatography is a method that separates molecules according to their size and shape. Gel filtration chromatography was used as an analytical method to determine the molecular weight of purified TAA.

Requirements

- Sephadex G-75.
- 50 mM Sodium phosphate buffer, 0.15 M NaCl, (pH 7).
- Markers: Ribonuclease A, Albumin (bovine serum), Ovalbumin, Trypsin.
- Blue dextran
- Potassium ferricyanide

Procedure

A calibration curve was prepared by measuring the elution volumes of several protein standards and plotting the logarithm of their molecular weight versus the ratio of their elution volume. The molecular weight of the purified TAA was determined from the calibration curve once its elution volume was measured. A fresh, filtered solution of Blue Dextran was prepared. This was applied to the column to determine the void volume (V_0), and to check the column packing. The selected calibration proteins were dissolved in the running buffer and applied to the column. The elution volumes (V_e) for the standards were determined

by measuring the volume of the eluent from the point of application to the centre of the elution peak. A calibration curve of the logarithm of their molecular weight versus V_0/V_e was prepared. The sample is applied in a volume <2% of the total column volume (V_t) and the elution volume (V_e) of the molecule of interest is determined. The corresponding molecular weight of the protein was from the calibration curve after determining its elution volume.

IMMUNIZATION OF THE ANIMALS

A group of 5 to 7 female mice of around 12 weeks weighing 25gm were immunized with the antigen to raise antibody.

Antigen preparation:

- Aqueous solution of purified TAA emulsified with Complete Freund's adjuvant (1:1).
- Aqueous solution of purified TAA emulsified with Incomplete Freund's adjuvant (1:1).

Procedure

A group of 5 to 7 mice, each around 12 weeks old received one intramuscular injection of the aqueous solution of purified TAA emulsified with Freund's complete adjuvant as primary immunization. The booster was administered with purified TAA formulation in incomplete Freund's adjuvant on the 30th day. Blood was collected from the animals by retro-orbital bleeding on the 3rd and 7th day after booster injection. The serum obtained was pooled and stored at 20°C.

WESTERN BLOT

Immunoblotting is a widely applied and well-established analytical method for the detection and analysis of protein in a test sample. By analysis of intensity and location of the specific reaction, expression details of the target proteins in the given cells or tissue homogenate can be obtained. Western blotting is a highly sensitive technique and can detect proteins as low as 1.0ngm in the test sample.

Requirements

- Towbin buffer: 25mM Tris, 195mM Glycine, 20% w/v Methanol, (pH8.3).
- TBS: tris buffer saline (pH7.5).
- TTBS: 0.5ml of Tween 20 in 1lit of TBS.
- Blocking buffer: 5% non-fat dry milk in TBS.
- Anti-mouse HRP conjugates.
- TMB/H₂O₂.

Procedure

SDS-PAGE was run and the SDS-PAGE gel and the nitrocellulose membrane were soaked in chilled Towbin buffer for about 30min. The foam pads and the four sheets of blotting paper were also soaked in chilled Towbin buffer. The blotting was done at a current of 300mA for 1hr in chilled Towbin buffer as running buffer. An ice pad was placed inside the assembly with constant stirring. After the completion of the run, the membrane was washed in TTBS thrice for 5 min each wash, followed by washing again in TBS thrice for 5min each. The membrane is then incubated with the blocking buffer at room temperature for 1 hr on a gel rocker. The membrane was then washed in TTBS thrice for 5 min each and incubated with the primary antibody (serum in 5% non-fat dry milk at a dilution of 1:1000) overnight. The membrane was washed with TTBS followed by TBS as described above. After washing, the membrane was incubated again with a secondary antibody (1:500 dilutions with non-fat dry milk) for 3hrs at room temperature. Washing was done again and the membrane was allowed to dry. TMB/H₂O₂ (diluted

1:20 in distilled water) was added to the membrane. The membrane was washed with distilled water when the blue band was observed and allowed to dry.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The presence of circulating antibodies against TTA in the immune and normal sera was determined using ELISA described by (Catty et al., 1989). It is a serological assay in which the bound antigen/antibody is detected by a linked enzyme that converts a colourless substrate into a coloured product.

Requirements

- Coating buffer: 0.05M Sodium carbonate/bicarbonate (pH 9.6)
- Washing buffer: 0.1M Phosphate buffer saline (PBS) containing 0.05% Tween 20 (pH 7.4)
- Blocking buffer: 5% Non-fat dry milk in washing buffer.
- Anti-mouse HRP conjugate.
- TMB/H₂O₂.

Procedure

Microtiter plate wells were coated with 50 µl of 1 µg/ml TAA in coating buffer. The plate was covered and incubated overnight at 4°C. The wells were then washed with washing buffer for 2-3 times. This was followed by blocking the wells with 100 µl of blocking buffer and incubated overnight at 4°C. After washing the wells again, 50 µl of serially diluted serum (in blocking buffer) was applied to each well of the plate and was incubated for 2 hr at 37°C followed by washing with washing buffer. The commercially supplied anti-mouse-HRP conjugate was diluted 1000 times (in blocking buffer) and 50 µl of it was added to each well and incubated for 2 hr at 37°C followed by washing step. 50 µl of the substrate (20 fold diluted in distilled water) was added and incubated in the dark for 10 min at room temperature. The reaction was stopped by adding 50 µl of 1M H₂SO₄. The absorbance of resulting yellow coloured product was read at 450 nm.

RESULT

After 16 weeks of treatment, marker enzyme test clearly indicated the pathogenesis of liver cancer. Histological examination showed that cells are in rapid state of division and support the progression of cancer induction (dutta et al 2016). After looking at the results of marker enzymes, liver function test and histological examination of the liver tissues it was confirmed that N-Nitrosodibutylamine (10mg/kg body weight) at a weekly interval for 16 weeks induces liver cancer. After successful induction of liver cancer by N-Nitrosodibutylamine our main target of interest was to see whether DBN affects the liver mitochondria and its membrane surface protein or not.

SDS-PAGE ANALYSIS OF THE ISOLATED MITOCHONDRIAL MEMBRANE PROTEIN AND IDENTIFICATION OF TAA

Total protein concentration was estimated in isolated mitochondrial membrane proteins extracts from both treated and control mice and it was seen that concentration of the protein was significantly higher as shown in Figure I and table I below.

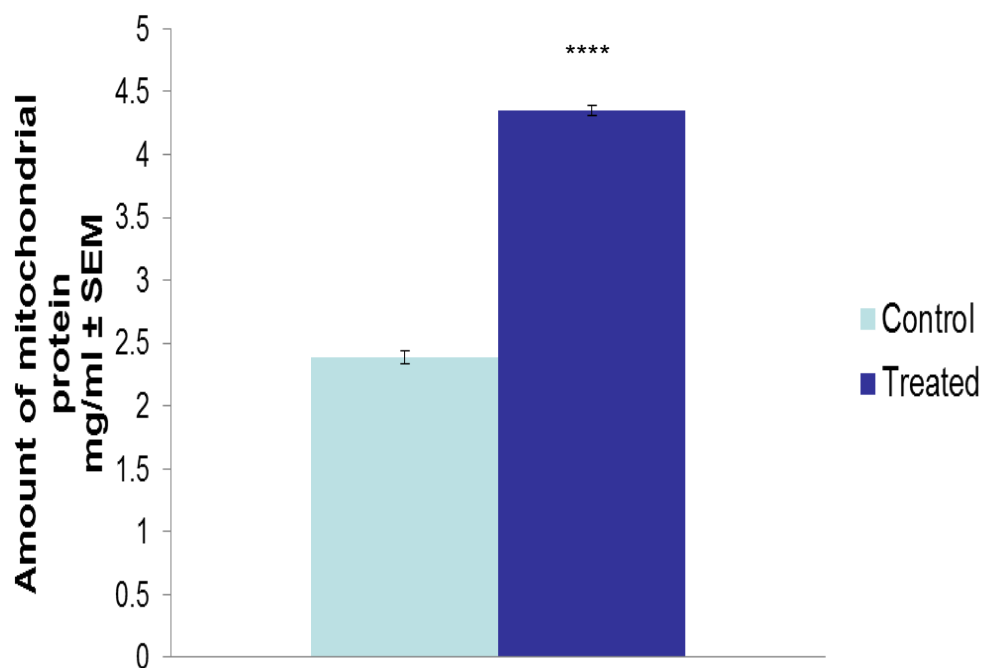


Fig. I Total mitochondrial membrane surface protein estimation. The values in the bars are expressed as Mean \pm SEM; n=10. ****p<0.0001 when control compared with treated

Table I Total mitochondrial membrane surface protein. n=10. ****p<0.0001 when control compared with treated

Groups	Amount of mitochondrial membrane surface protein mg/ml \pm SEM, n=10
Control	2.39 \pm 0.057
Treated	4.35 \pm 0.040

Isolated mitochondrial membrane proteins extracts from both treated and control mice were analysed on the SDS-PAGE. Proteins were resolved into several protein bands on a 15% SDS-PAGE followed by CBB staining of the gel. Differential expression of proteins was observed in the liver mitochondrial membrane of treated mice from those of control Figure II. A protein of approximately 14kDa was found to be prominently overexpressed in treated mice which were also seen in normal mice, as seen clearly from the intensity of the bands in both control and treated mice. In the control mice, the protein was found to be expressed in less intensity as compared to its higher intensity band in the treated mice.

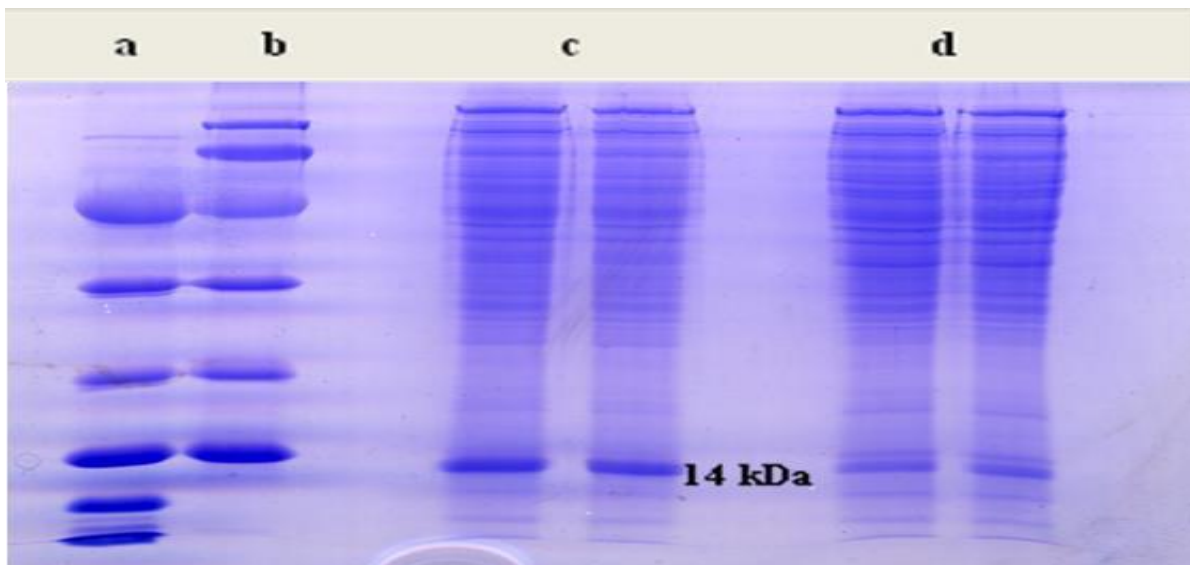


Fig. II SDS-PAGE analysis of the extracted mitochondrial membrane surface protein obtained from N-Nitrosodibutylamine (DBN) treated mice and normal control mice. Lane (a) Low weight molecular marker, Lane (b) Medium weight molecular marker, Lane (c) DBN treated extract and Lane (d) Normal control mice. A protein of approximately 14 kDa was seen to be overexpressed in the lane 3 containing DBN treated extract

SEPERATION PURIFICATION OF TAA

ION EXCHANGE CHROMATOGRAPHY

After identification of the over-expressed protein which was considered as TAA from the treated mice by SDS-PAGE analysis, the extract was subjected to cation exchanger CM- Sephadex C50 for the separation and purification. The elution profile of the cation exchange chromatography is shown in Figure III. It was seen that most of the proteins eluted out with 50mm Phosphate buffer pH 7 in the unbound fraction Figure III Peak 1, 2. Some minor peaks were also seen in the bound region. The fractions were pooled separately, concentrated and re-resolved on an SDS-PAGE to identify the presence of the desired 14kDa protein.

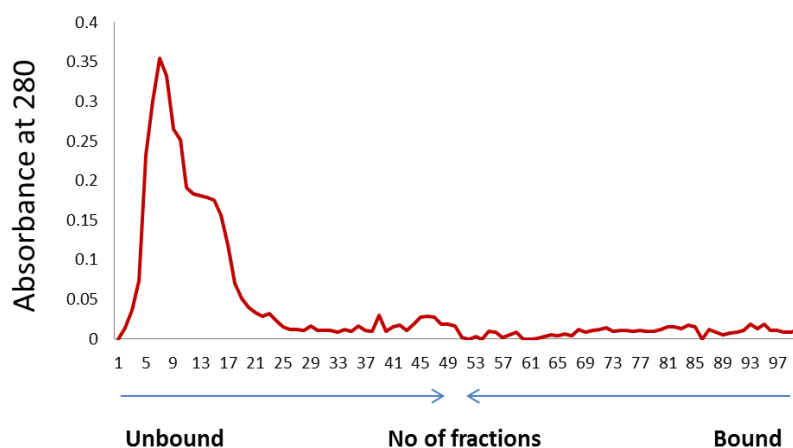


Fig. III CM-Sephadex C50 a cation exchange protein profile of the liver mitochondria membrane surface protein from DBN treated mice

SDS-PAGE ANALYSIS OF THE PURIFIED PROTEIN

It was confirmed by SDS-PAGE analysis that the protein of our interest eluted out in the unbound fraction as seen in Figure IV and it was anionic in character. The identified 14 kDa protein was found to absent in the bound region i.e. peak 3, 4 of the elution profile. A prominent band of approximately 14 kDa was observed in the lane loaded with the fractions of the unbound region.

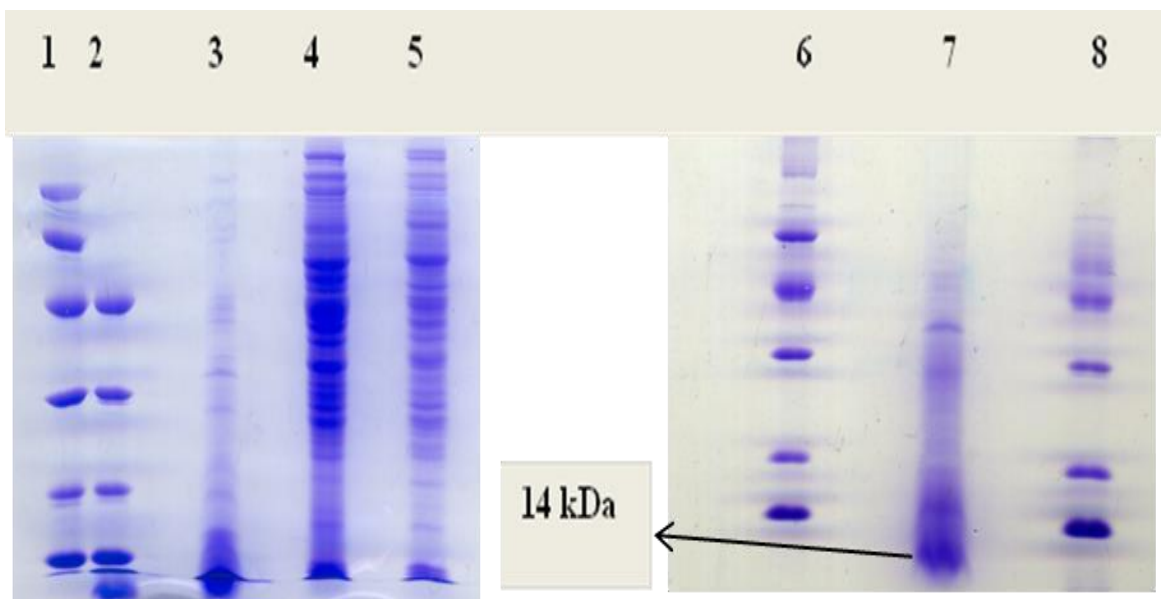


Fig. IV SDS-PAGE analysis of the unbound fractions obtained from the cation exchange column chromatography of the DBN treated extract. Lane 1 and 6: Medium weight molecular marker, Lane 2 and 8: Low weight molecular marker, Lane 3 and 7: Unbound fractions, Lane 4: DBN treated extract and Lane 5: Control

PREPARATIVE SDS-PAGE FOR PURIFICATION OF THE PROTEIN

As we didn't obtain a single band from the ion exchange chromatography an additional purification step was carried out. The fractions corresponding to the peaks were pooled together, dialysed against distilled water, concentrated and further purified using preparative SDS-PAGE. The TAA recovered from the preparative SDS-PAGE was subjected to an SDS-PAGE gel and a single band was obtained as shown in Figure V.

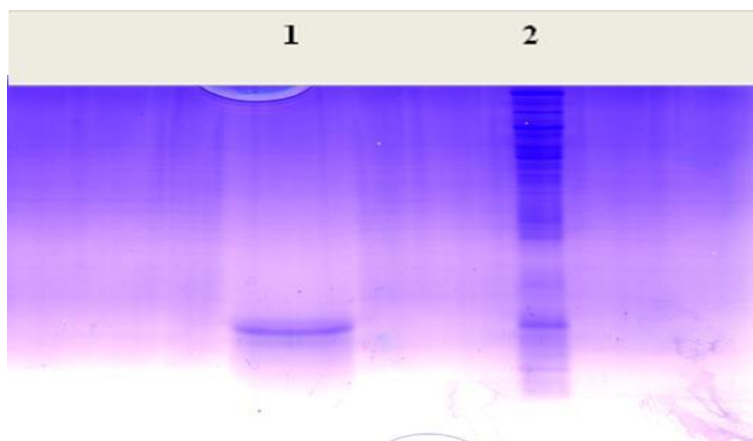


Fig. V Preparative SDS-PAGE purified TAA. The portion of the gel corresponding to the TAA was cut out of the unstained gel. It was cut into small pieces and homogenized in 3% 1- butanol. The homogenate was centrifuged at 8000 g for 30 min at 4°C. The supernatant collected was pooled and dialyzed. Lane 1: Purified TAA and Lane 2: DBN treated extract

MOLECULAR WEIGHT DETERMINATION

The identified and purified protein was then subjected to gel filtration chromatography in order to determine the molecular weight of the purified protein. The molecular weight of the purified protein was determined from the plot of log molecular weight versus V_e/V_o of several calibration standards such as Trypsin, Ribonuclease A, Albumin (bovine serum), and Ovalbumin. The logarithm of their respective molecular weight was plotted against the ratio of their elution volume to void volume. From the calibration curve, the molecular weight of the purified protein was found to be 14.15 kDa Fig VI.

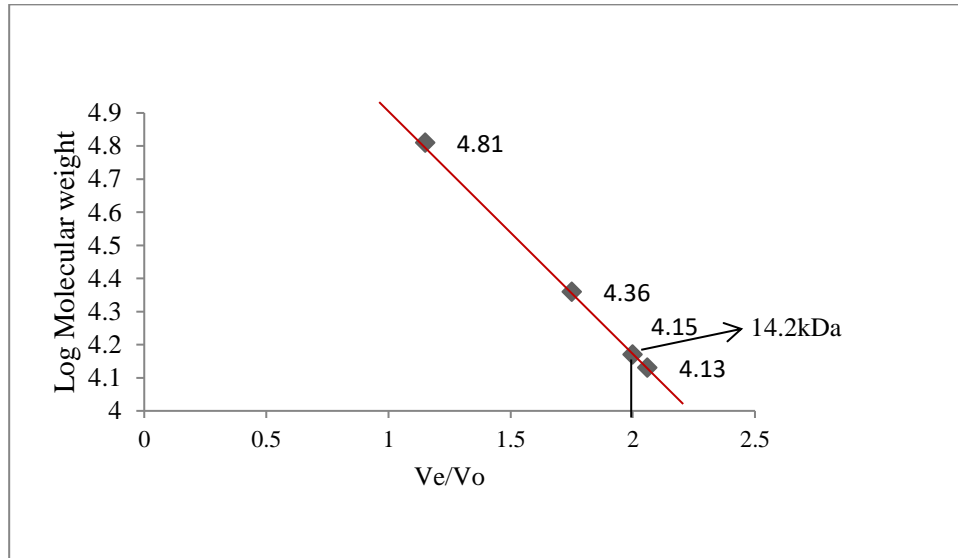


Fig VI Sephadex G-75 Gel filtration profile showing the molecular weight of the fraction obtained by cation-exchange chromatography.

IMMUNOGENECITY OF THE TAA

Immunogenicity of the purified TAA was checked in control mice. Mice immunized with the formulation of TAA were used for the carrying out the immunogenicity test. Anti-sera obtained from the immunized were tested for the presence of anti-TAA antibody using

- Western blotting and
- Enzyme linked immune adsorbent assay (ELISA).

WESTERN BLOTTING

This technique was used to detect the presence of antibodies against TAA in the serum collected from the mice immunized with TAA formulation. Appearance of the single band as shown in the Figure VII confirmed the presence of anti-TAA antibodies in the serum of the immunized mice.

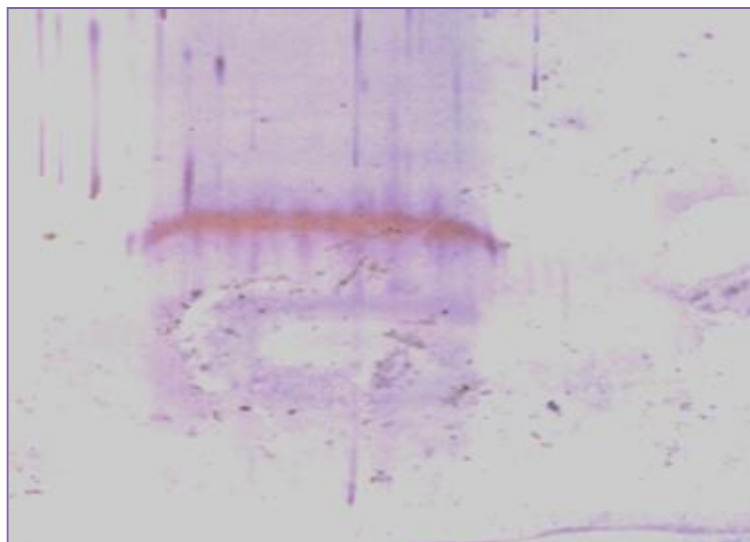


Fig VII The immunoblots of normal mice treated with Adjuvant and TAA formulations

ENZYME LINKED IMMUNOSORBENT ASSAY

The anti-TAA antibody titers were determined by ELISA in the serum obtained from immunized animals. The results of ELISA have been shown in the Figure VIII and Table II below. The results indicate clearly that the purified TAA is immunogenic and may evoke an antibody response upon its immunization.

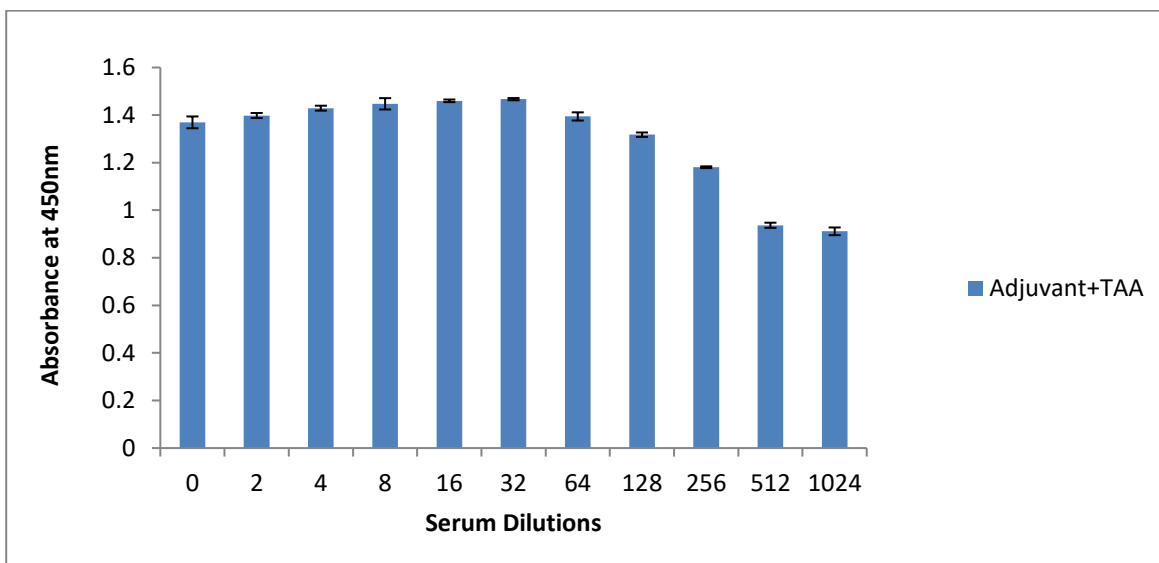


Fig VIII Bar diagram showing high anti-TAA antibody level at different dilution of serum from mice immunized with TAA – Adjuvant formulation. The values are expressed as MEAN±SEM, n=10

Table II Anti-TAA antibody level at different dilution of serum from mice immunized with TAA – Adjuvant formulation. The values are expressed as MEAN±SEM, n=10.

Dilution	Adjuvant +TAA
0	1.36946 ± 0.024871

2	1.39822 ± 0.010608
4	1.42913 ± 0.010313
8	1.44741 ± 0.023867
16	1.460048 ± 0.005512
32	1.46696 ± 0.004584
64	1.3944 ± 0.017219
128	1.317559 ± 0.009368
256	1.1807 41± 0.00372
512	0.93673 ± 0.010867
1024	0.911386 ± 0.016227

DISCUSSION

N-Nitrosodibutylamine (DBN) belongs to the nitrosamines family and is considered as a potential environmental carcinogen. In the present study, its effects on the liver mitochondria in swiss albino mice have been examined. It was seen in our present investigation that weekly intravenous administration of DBN (10 mg kg⁻¹ body weight) in 5% ethanol up to a period of 16 weeks induces hepatocarcinogenesis in Swiss albino mice.

An attempt was made for proteomic analysis of the liver mitochondrial membrane surface proteins in DBN-treated animals. The SDS-PAGE analysis of liver mitochondrial membrane protein extract showed differential expression of membrane surface proteins in mice upon DBN exposure as compared to that of the normal control (Fig II). Several proteins were found up-regulated or over-expressed upon comparison with the normal. The observed alteration in protein expression clearly indicates that DBN has inflicted major changes in the membrane of mitochondria. These changes could be involved in causing distortions and alterations of the mitochondrial membrane during DBN treatment. On doing SDS-PAGE analysis a protein of approximately 14kDa was found to be overexpressed in the mitochondrial extract from DBN treated mice when compared to that of the extract from control mice. This overexpressed protein was also present in the control mice but was found in trace amount as shown in Figure. We considered this over-expressed as tumor-associated antigen (TAA) because as mentioned above this protein was also present in normal mice but was over-expressed in the case of the DBN treated mice.

After the identification of the TAA an attempt was then made to separate and purify the over-expressed protein which we considered as TAA in DBN treated mice for which ion exchange chromatography, preparative SDS-PAGE, gel filtration methods were employed. On doing the cation exchange chromatography using CM-Sephadex C 50 a cation exchanger it was found that the most of the protein had eluted out in the unbound fractions as we can see in the Fig.III whereas some minor peaks were also seen in the bound region. So to confirm whether the protein of interest had eluted out in bound or unbound region SDS-PAGE analysis of both bound and unbound fractions were done. On doing SDS-PAGE analysis the TAA was seen to be eluted out in the unbound fraction. The desired glycoprotein was found to be an anionic protein as evident from the elution profile shown in Fig IV.

Further purification of the glycoprotein was carried out by gel filtration chromatography using Sephadex G-75, but could not be achieved due to small differences in the molecular weight of other anionic protein eluted with it. Hence was finally purified by preparative SDS-PAGE. TAA extracted from the preparative SDS-PAGE method was subjected to SDS-PAGE to check its homogeneity and purity. TAA was found to be homogeneous and appeared as a single band (Fig.V).

The molecular weight of TAA was determined by gel filtration chromatography. Sephadex G 75 column was used for this purpose. The calibration curve was prepared using protein molecular weight markers such as albumin, trypsin, and ribonuclease A. The logarithm of their respective molecular weights (Log Mr) was plotted against the ratio of their elution volume to void volume (V_e / V_o). From the calibration curve, the molecular weight of the purified glycoprotein was read and found to be very close to 14.2 kDa (Fig.VI).

The purified TAA was tested for its immunoreactivity in allogeneic normal control Swiss albino mice through active immunization using TAA-formulations as discussed in the method section. The level of antibody raised in mice upon immunization was first analysed using western blot technique. The appearance of a prominent single band as depicted in Fig.VII for antigenic formulation signifies the high antibody concentration against TAA in test serum. The anti-TAA antibody concentrations in immune sera were also determined by ELISA. The antibody titers were found significantly very high against the formulation i.e. TAA emulsified with CFA as has been shown in Fig.VIII. These observations clearly indicate that the purified TAA is an anionic glycoprotein with very high content of carbohydrate moieties, highly immunogenic, may elicit a significantly high antibody response against it in mice upon immunization and thus could suitably be used as an effective target for active immunotherapy against cancer.

CONCLUSION

Our study revealed that DBN not only make alterations in hepatocytes but also induces significant alterations in the morphology, functional aspects of liver mitochondria as well as in the differential expression of the membrane surface proteins. These changes in mitochondria provide a unique potential for the use of mitochondria as markers for the early detection for the treatment of liver cancer.

DECLERATIONS

Funding: North Eastern Hill University.

Conflicts of interest/Competing interests: None. Availability of data and material: NA.

Code availability: NA

Ethics approval (In case animals were involved): All applicable international, national, and/or institutional guidelines for the care and use of animals were followed, for using swiss albino mice.

Ethics approval (In case humans were involved): NA

Consent to participate: NA.

Consent for publication: I, the undersigned, give my consent for the publication of identifiable details, which can include photograph(s) and/or case history and/or details within the text ("Material") to be published in the above Journal and Article.

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