Protective Effect of Allyl Isothiocyanate on Glycoprotein Components in 7,12-dimethylbenz(a)anthracene Induced Mammary Carcinoma in Rats

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Abstract The present study aimed to investigate the protective effect of allyl isothiocyanate (AITC) on glycoprotein components in 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis in female Sprague–Dawley rats. Mammary tumor was induced by a single dose of DMBA (25 mg/rat) injected subcutaneously near mammary gland. The levels of glycoprotein components such as hexose, hexosamine and sialic acid were analyzed colorimetrically in plasma, mammary and liver tissues. We observed an increased levels of glycoprotein components in plasma, mammary and liver tissues in cancer bearing rats. It was further confirmed by Periodic Acid Schiff staining in mammary and liver tissues. Upon oral administration of AITC to DMBA injected rats, the abnormal changes were reverted back to near normal levels and biochemical findings are supported by histological analysis. This could be due to the anti-neoplastic potential of AITC against DMBA-induced mammary carcinogenesis. The result shows that AITC has the potential to inhibit abnormal glycosylation that favors neoplastic transformation.

Keywords Allyl isothiocyanate · 7,12-dimethylbenz(a)-anthracene · Glycoprotein components · Mammary carcinogenesis

Introduction

Breast cancer, most common neoplasm in women and is leading cause of cancer related death worldwide [1]. Neoplasm starts by slight molecular changes within a cell, which can be divided into three different stages: initiation, promotion and progression [2]. In each stage, various genetic alterations that gradually occur in the cells leads to metastasis and finally cause mortality. However, malignant neoplastic changes due to distant metastasis do not respond to cancer therapy. In order to metastasize, cells migrate and invade into the surrounding tissue, survive in circulation, intravasate into the blood vessel or lymphatic system, extravasate and finally anchor and proliferate at a distant site [3].

Glycoproteins are conjugated proteins in which carbohydrate moieties are connected via covalent linkage. Glycoproteins play a vital role in cell-to-cell interaction, intracellular processing of proteins, cell activation, adhesion and metastasis [4]. Glycoproteins are released from cancer cells to blood circulation during pathogenesis [5]. Changes in carbohydrate moieties of glycoproteins have been linked to play an important role during neoplastic transformation, which is associated with changes in tumor cell surface and altered cell surface carbohydrate moieties of the cell membrane due to proliferation, invasion and metastasis [6, 7]. Thus, the combined appraisal of hexose, hexosamine and sialic acid components of glycoproteins are useful in strengthening the monitoring of diagnosis and treatment of breast cancer survivors [8, 9].

Allyl isothiocyanates (AITC) is a naturally occurring isothiocyanates of organosulfur compound, which is present in cruciferous vegetables. The anti-tumor and anti-cancer activities of AITC are due to inhibition of tumor formation and cell metastasis by inhibition of cell

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adhesion, migration and invasion [10, 11]. Hence, the present study was designed to investigate the protective effect of AITC on glycoprotein components in DMBA-induced mammary carcinoma in rats.

Materials and Methods

Chemicals

AITC and DMBA were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. All the other chemicals used were of analytical grade.

Animal Model

Adult female Sprague–Dawley rats (weighed 100–120 g) at the age group of 6 and 7 weeks old were obtained from National Institute of Nutrition, Hyderabad, India. The rats were maintained in the controlled environmental conditions of temperature (24 ± 2 °C) and humidity ($50 \pm 10\%$) on alternative 12 h light/dark cycles in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University, Chidambaram, Tamilnadu, India. The rats were fed with standardized rat pellet and water ad libitum. The experiment was approved by the Institutional Animal Ethics Committee (IAEC), regulated by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) (Reg No. 160/1999/CPCSEA and Proposal No. 983).

Dose Fixation

The effective dose of AITC was fixed in my previous study. Preliminary biochemical and histological studies were shown remarkable response at the concentration of 20 mg/kg bw [12]. Hence, this concentration was selected for this study.

Experimental Design

After dose fixation, the total number of 40 rats were randomly divided into four groups and each group contains 10 rats. Mammary carcinogenesis was induced by a single subcutaneous injection of 25 mg of DMBA in 1 mL emulsion of sunflower oil (0.75 mL) and physiological saline (0.25 mL) to each rat [13].

Group 1: Normal control (vehicle treated)

Group 2: DMBA (25 mg/rat)

Group 3: DMBA+AITC (20 mg/kg bw dissolved in 1 mL corn oil)

Group 4: AITC (20 mg/kg bw dissolved in 1 mL corn oil).

Biochemical Analysis

Collection of Blood and Tissues

At the end of 16th week, rats were fasted overnight and sacrificed by cervical decapitation. Blood samples were collected in heparinized tubes and the plasma used for the biochemical analysis. Mammary and liver tissues were excised immediately from the rats and stored in ice-cold containers. Tissues were homogenized with suitable buffer, centrifuged at 3000 g for 10 min and the supernatant were used for biochemical estimations on the same day of sacrifice. Mammary and liver tissues were preserved in 10% formalin and stored at -80 °C for histological analysis.

Extraction of Glycoproteins

To 0.1 mL of plasma, 5.0 mL of methanol was added, mixed well and centrifuged for 10 min at 3000 g. The supernatant was decanted and the precipitate was again washed with 5.0 mL of 95% ethanol, recentrifuged and the supernatant was decanted to obtain the precipitate of glycoproteins which was used for the estimation of glycoprotein components [14].

Tissues were defatted by the method of Folch et al. [15] for the estimation of glycoproteins. A known weight of the tissue was homogenized in 7.0 mL of methanol. The contents were filtered and homogenized with 14.0 mL of chloroform. This was filtered and the residue was successively homogenized in chloroform–methanol (2:1, v/v) and each time the extract was filtered. The residue (defatted tissues) was obtained and the filtrate decanted. A weighed amount of defatted tissue was suspended in 3.0 mL of 2 N HCl and heated at 90 °C for 4 h. The sample was cooled and neutralized with 3.0 mL of 2 N NaOH. Aliquots from this were used for estimation of hexose, hexosamine and sialic acid.

Determination of Glycoproteins

Hexose was estimated by the method of Niebes [16]. The reaction mixture contained 0.5 mL of plasma/tissue homogenate, 0.5 mL of 5% phenol and 2.5 mL of concentrated H_2SO_4 and was boiled for 20 min and absorbance was read at 490 nm.

Hexosamine was estimated by the method of Elson and Morgan [17] with slight modifications by Niebes (14). Briefly, the reaction mixture contained 0.5 mL plasma/1.0 mL tissue homogenate and 2.5 mL of 3 N HCl. It was boiled for 6 h and neutralized with 6 N NaOH. To 0.8 mL of the neutralized sample was added 0.6 mL of acetyl acetone reagent and boiled for 30 min. The mixture was

treated with 2.0 mL of Ehrlich's reagent. The color developed was read at 540 nm colorimetrically.

Sialic acid was determined by the method of Warren [18]. In brief, 0.5 mL of plasma/tissue homogenate was treated with 0.5 mL of de-ionized water and 0.25 mL of periodic acid and incubated at 37 °C for 30 min. Then, 0.2 mL of sodium meta-arsenate and 2.0 mL of thiobarbituric acid were added to the reaction mixture which was heated for 6 min, 5.0 mL of acidified butanol was then added and absorbance was read at 540 nm.

The hexose, sialic acid and hexosamine released from glycoproteins are expressed as mg/dL for plasma and mg/g defatted tissue for tissues.

Histological Analysis

For histological analysis, mammary and liver tissues were excised and immersed in 10% formalin solution for fixation, dehydrated with graded ethanol solutions from 50 to 100% and then embedded in paraffin. Paraffin-embedded mammary and liver tissue Sects. (3–5 µm) were cut using a microtome then rehydrated with xylene and graded series of ethanol. Glycoproteins in the mammary and liver tissues was analysed by Periodic Acid Schiff (PAS) base staining according to the method of Yamabayashi [19]. Images were captured by Nikon Coolpix 4500 microscope at 40× magnification. Quantify mammary and liver tissues glycoproteins by using standard quantification software ImageJ.

Statistical Analysis

Statistical analysis was performed using SPSS 16 (SPSS, Inc., Chicago) statistical package. The data are expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) comparison method was used to correlate the difference between the variables. Data are considered statistically significant if *p* values are less than 0.05.

Results

Effect of AITC on Liver Weight Changes in Control and Experimental Rats

Table 1 shows the liver weight of control and experimental rats. We observed significantly (*p* < 0.05) decreased in the liver weight of DMBA-induced tumor bearing rats when compared with the control group. On the contrary, treatment with AITC prevented the significant (*p* < 0.05) decrease in the liver weight of DMBA-induced tumor bearing rats. AITC alone group rats showed an increase in

Table 1 Effect of AITC on liver weight changes in control and experimental rats

Groups	Liver weight (g)
Control	7.8 ± 0.5 ^a
DMBA	4.5 ± 0.3 ^b
DMBA+AITC	7.5 ± 0.5 ^a
AITC	7.7 ± 0.5 ^a

Values are expressed as mean ± SD for ten rats in each group

Values not sharing a common superscript differ significantly at *p* < 0.05 (DMRT)

^a Comparisons were made between groups I, III and IV

^b Comparisons were made between group II

Table 2 Effect of AITC on plasma glycoprotein components in control and experimental rats

Groups	Hexose (mg/dL)	Hexosamine (mg/dL)	Sialic acid (mg/dL)
Control	136.7 ± 9.8 ^a	53.3 ± 3.5 ^a	58.8 ± 3.4 ^a
DMBA	185.9 ± 14.5 ^b	83.1 ± 6.0 ^b	93.1 ± 7.9 ^b
DMBA+AITC	145.2 ± 9.0 ^a	57.1 ± 3.2 ^a	63.2 ± 3.8 ^a
AITC	137.7 ± 9.9 ^a	54.6 ± 3.2 ^a	60.9 ± 4.2 ^a

Values are expressed as mean ± SD for ten rats in each group

Values not sharing a common superscript differ significantly at *p* < 0.05 (DMRT)

^a Comparisons were made between groups I, III and IV

^b Comparisons were made between group II

their liver weight, but no significant levels when compared with control rats.

Effect of AITC on Plasma Glycoprotein Components Levels in Control and Experimental Rats

Table 2 shows the plasma glycoprotein components levels of control and experimental rats. There was a significant increase in plasma glycoprotein components levels in cancer-bearing rats when compared to normal control rats. Administration of AITC to DMBA injected rats resulted in significant reduction of glycoprotein components levels in plasma, which was comparable with cancer-bearing rats. However, there was no change in AITC alone treated rats when compared with normal control rats.

Effect of AITC on Mammary Tissue Glycoprotein Components Levels in Control and Experimental Rats

Table 3 shows the mammary tissue glycoprotein components levels of control and experimental rats. There was a significant increase in mammary tissue glycoprotein

Table 3 Effect of AITC on glycoprotein components in mammary tissues of control and experimental rats

Groups	Hexose (mg/g defatted tissue)	Hexosamine (mg/g defatted tissue)	Sialic acid (mg/g defatted tissue)
Control	2.2 ± 0.2 ^a	1.4 ± 0.1 ^a	1.1 ± 0.1 ^a
DMBA	3.9 ± 0.3 ^b	2.9 ± 0.2 ^b	2.6 ± 0.1 ^b
DMBA+AITC	2.3 ± 0.2 ^a	1.5 ± 0.1 ^a	1.2 ± 0.1 ^a
AITC	2.2 ± 0.1 ^a	1.4 ± 0.1 ^a	1.2 ± 0.1 ^a

Values are expressed as mean ± SD for ten rats in each group

Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT)

^a Comparisons were made between groups I, III and IV

^b Comparisons were made between group II

Table 4 Effect of AITC on glycoprotein components in liver tissues of control and experimental rats

Groups	Hexose (mg/g defatted tissue)	Hexosamine (mg/g defatted tissue)	Sialic acid (mg/g defatted tissue)
Control	3.7 ± 0.2 ^a	3.1 ± 0.2 ^a	2.0 ± 0.1 ^a
DMBA	6.0 ± 0.4 ^b	4.8 ± 0.3 ^b	3.9 ± 0.2 ^b
DMBA+AITC	3.8 ± 0.3 ^a	3.3 ± 0.3 ^a	2.1 ± 0.1 ^a
AITC	3.7 ± 0.2 ^a	3.1 ± 0.2 ^a	2.1 ± 0.1 ^a

Values are expressed as mean ± SD for ten rats in each group

Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT)

^a Comparisons were made between groups I, III and IV

^b Comparisons were made between group II

components levels in cancer-bearing rats when compared to normal control rats. Administration of AITC to DMBA treated rats resulted in significant reduction of glycoprotein components levels in mammary tissue, which was comparable with cancer-bearing rats. However, there was no change in AITC control rats when compared with normal control rats.

Effect of AITC on Liver Tissue Glycoprotein Components Levels in Control and Experimental Rats

Table 4 shows the liver tissue glycoprotein components levels of control and experimental rats. There was a significant increase in liver tissue glycoprotein components levels in cancer-bearing rats when compared to normal control rats. Administration of AITC to DMBA treated rats resulted in significant reduction of glycoprotein components levels in liver tissue, which was comparable with cancer-bearing rats. However, there was no change in AITC control rats when compared with normal control rats.

Histological Changes in Mammary Tissue

Figure 1a–d shows the PAS staining for the glycoproteins of mammary tissues of control and experimental rats. The group II (b) cancer-bearing rats showed significantly increased glycoproteins, which is reduced in group III (c) AITC treated rats. The group I (a) controls and group IV (d) AITC alone treated rats exhibited very low accumulation of glycoprotein levels.

Histological Changes in Liver Tissue

Figure 2a–d shows the PAS staining for the glycoproteins of liver tissues of control and experimental rats. The group II (b) cancer-bearing rats showed significantly increased glycoproteins, which is reduced in group III (c) AITC treated rats. The group I (a) controls and group IV (d) AITC alone treated rats exhibited very low accumulation of glycoprotein levels.

Discussion

Glycoproteins are carbohydrate-protein complexes in which oligosaccharides moieties are covalently linked to particular amino acids of proteins. The important sugar moieties in oligosaccharides are hexoses, hexosamine and sialic acid. The glycoprotein components levels in serum and tissues have been associated with the status of the cancer. Glycoproteins are major constituents of cell membranes, plays vital role in cell–cell interaction, cell differentiation, cell proliferation, carcinogenesis and malignant transformation [20].

Proteins bound hexose act as markers of carcinogenic process and contributes hydrophilic nature to the cell membrane [21]. An increased level of hexose plays an important role in maintaining the structure, rigidity and function of cell membrane [22]. In this study, we observed an increased level of hexose in DMBA treated rats. Increased level of hexose in cancer bearing rats was also reported in previous study [23]. AITC treatment decreases hexose level in plasma, mammary and liver tissues indicate the efficiency of maintaining cell integrity.

Hexosamine in glycoprotein contributes its cationic charges and makes the cell membrane polarized [21]. In our study, DMBA treated rats showed increased hexosamine in plasma and tissues of cancer bearing rats. Purushothaman et al. [24] also reported that elevated level of hexosamine in cancer bearing rats, this may be due to the damage of connective tissues in mammary tumor [25]. However, AITC administration significantly decreases hexosamine level that indicating its antimetastatic property.

Fig. 1 PAS staining in the mammary tissues of control and experimental rats (a–d). DMBA treated rats (b) showed significantly increased glycoproteins, which were altered in AITC treated rats (c). Controls (a) and AITC alone treated rats (d) exhibited very low accumulation of glycoprotein levels. 40× magnification

Sialic acid is present as terminal sugar in glycoproteins, which impart a net negative charge to the cell surface and are essential in cell-to-cell and cell-to-matrix interactions [26]. It was previously documented that neoplastic transformation leads to increased plasma sialic acid concentration due to shedding or secretion of sialic acid from the tumor cells [27, 28]. Elevated level of sialic acid useful for early detection of cancer, prognosis of the disease, degree of metastasis and recurrence. Treatment of AITC to rats significantly inhibit the increasing levels of sialic acid, this might be due to its anti-tumor activity.

PAS staining is an indicator for glycoprotein, which is present in the basement membranes, including those of blood vessels and tumor cells involved in the neoplastic process and distant metastasis [29]. Increased PAS staining shows the presence of high glycoprotein

content in tumor tissue, which is well in accordance with the previous findings of Arivazhagan and Sorimuthu Pillai [30]. High intensity PAS staining shows significantly increased in cancer conditions, this may be due to the cancer cells preparing itself for metastasis [30].

AITC significantly alters the expression of glycosyltransferases thereby reduce glycoprotein synthesis and protected the structural integrity of cell surface and membrane, indicating its potent anticancer property. Since AITC has already been demonstrated to inhibit tumor growth, through the alteration of glycoprotein levels in N-nitrosodiethylamine induced liver carcinogenesis [31]. In our previous study also reported that AITC significantly prevented the tumor growth and volume in DMBA treated rats [12]. The results clearly indicating the tumor inhibitory or anti-tumor activity of AITC.

Fig. 2 PAS staining in the liver tissues of control and experimental rats (**a–d**). DMBA treated rats (**b**) showed significantly increased glycoproteins, which were altered in AITC treated rats (**c**). Controls (**a**) and AITC alone treated rats (**d**) exhibited very low accumulation of glycoprotein levels. 40× magnification

Conclusion

We conclude that AITC has the ability to suppress malignancy by modulating the cell transformation, decreasing the degree of metastasis and inhibiting the progression by causing effective changes in the structure of cell membranes. This could be due to the anti-neoplastic, cytostabilizing potential and also inhibitory action of AITC against carcinogenesis. Further, it should be confirmed by molecular studies of proteins involved in glycosylation.

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Compliance with Ethical Standards

Conflict of interest The first author (Mr. T. Rajakumar) has received Senior Research Fellowship (SRF), from Indian Council of Medical Research (ICMR), Government of India. The corresponding author (Dr. P. Pugalendhi) and last author (Mrs. S. Thilagavathi) declares that there are no conflicts of interest.

Ethical Approval This study was approved by the Institutional Animal Ethics Committee (IAEC), regulated by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) (Reg. No. 160/1999/CPCSEA and Proposal No. 983).

Informed Consent This article does not contain any studies with human participants.

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