Arsenic Induced Gallbladder Cancer Risk in Eastern Part of Indo-Gangetic Plains

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Abstract

Background

In recent times Gallbladder cancer (GBC) incidences increased many folds in India. Majority of GBC cases are being reported from arsenic hotspots identified in Bihar.

Methods

In this prospective study volunteers were selected who underwent surgery in our cancer institute. There were 11 control benign gallbladder cases and 28 confirmed gallbladder cancer cases. Their biological samples such as blood, gallbladder tissue, gallbladder stone, bile and hair samples were collected for arsenic estimation. Moreover, n=512 gallbladder cancer patients blood samples were evaluated for the presence of arsenic to understand exposure level in the population.

Results

A significantly high arsenic concentration ($p < 0.05$) was detected in the blood samples, gallbladder tissue, gallstones, bile and hair samples in comparison to the control group. Moreover, n=512 blood samples of GBC patients had significantly very high arsenic concentration.

Conclusions

The study shows very high arsenic concentration observed in the blood, gallbladder tissue, gallbladder stone, bile and hair samples in GBC cases in comparison to the benign control cases indicates the correlation between chronic arsenic exposure and gallbladder cancer incidences in eastern Indo-Gangetic plains region. The study also makes an attempt to establish the likely correlation/association between arsenic exposure and gallbladder cancer disease.

Introduction

Gallbladder cancer (GBC) is one of the rarest biliary tract malignancies with high mortality rate with relatively less survivability of 5 years.¹⁻² Globally in year 2020, out of total cancer cases of 19,292,789, new GBC cases reported in the year was 115,949 while number of deceased cases were 84,695.³ India accounts for 10% of global GBC cases, about one million new cancer cases every year with mortality rate as high as 33% every year.⁴ According to Indian Council of Medical Research (ICMR), it is estimated that by the year 2025, there will be 5-fold increase in cancer incidences in India, in which there will be 2.8-fold increase due to tobacco while 2.2-fold increase due to ageing. Such a drastic increase in cancer cases is more than the double that is predicted for the developed countries like USA.⁵⁻⁷

GBC has a very unusual geographical distribution with high incidences seen in pockets as high incidences are seen in Chile, India, Japan, Poland, Israel, Bolivia, Thailand, South Korea etc.⁸⁻¹¹ In India, its highest distribution has been seen in the states like Uttar Pradesh, Bihar, Orissa, West Bengal, Assam and

Delhi (National Capital Territory).¹²⁻¹⁵ The cancer is two folds more common in women in comparison to men.¹⁶ Population based data reveals that the incidences of these disease is very high in the northern cities (22 per 100,000) and low (0-0.7 per 100,000 women) in southern India.¹⁷⁻²⁰ The present institute caters the greatest number of cancer patients from the state of Bihar for the diagnosis and the treatment. Among the total cancer patients, 8.3% and 16.9% are GBC cases in male and female, respectively.⁵ GBC patients exhibit various clinical symptoms, including benign biliary disease on one hand and incurable malignant on other hand.²¹⁻²³ The etiology of gallbladder cancer apart from the few reasons has been a mystery, however the interplay of gallstones, genetic susceptibility, changes in the lifestyle factors and infections lead to progression of the cancer disease.²⁴⁻²⁵

Environmental pollutants in the recent times have become serious health hazards to the population. Due to ever increasing anthropogenic activities the level of the pollution effect has increased manifold leading to eruption of the diseases in the population.²⁶ Human exposure to arsenic a non-essential metalloid, has increased in recent times due to geogenic changes and anthropogenic activities which has caused severe health hazards in populations around the world.²⁷⁻²⁹ The inorganic arsenic when enters the human body is metabolized to organic arsenic (Monomethylarsonous acid- MMA(III), dimethylarsinic acid- DMA (V) and trimethylarsine TMA) which is still a toxic carcinogen. These organic compounds bind with DNA molecules which can influence transcription and translation leading to cancer. However, it is eliminated by the body as metabolic wastes via kidney. It also gets deposited in the keratin containing tissues like hair, nail and skin.³⁰ Arsenic is known to be carcinogen as (carcinogen category- I) in all the forms – arsenic trioxide, arsenic pentoxide, arsenous acids, arsenic acids and their salts (arsenites and arsenates).³¹⁻³⁵ Humans are exposed to this inorganic arsenic mainly by two ways – consumption of arsenic contaminated water and food.³⁶⁻³⁹

Arsenic is a known to cause cancer of skin, bladder, lungs, kidney, liver and prostate.⁴⁰⁻⁴² Arsenic causes cellular toxicity by inducing oxidative stress (ROS) which in turn leads to genotoxicity.⁴³ The continuous exposure of arsenic creates a very high oxidative that eventually leads to the appearance of symptoms like arsenicosis, skin lesions, blackfoot disease, vascular disease, hormonal imbalance and cancer.⁴⁴⁻⁴⁶ Due to its physical characters (no odour, colour or flavour), arsenic exposure is often unnoticed until people develop some arsenicosis symptoms due to drinking of arsenic contaminated water. The World Health Organisation (WHO) and the U.S Environmental Protection Agency (USEPA) have recommended a threshold of 10µg/L for inorganic arsenic concentration in drinking water. Unfortunately, millions of people are exposed to much higher toxic levels of arsenic and some populations are unaware of its ill effects, which in long term is causing the development of malignancies. Various molecular pathways, which has been deciphered in recent times, shows the progression of the disease by arsenic biotransformation process, that alters the methylation pattern, which is thought to play a key role in its carcinogenicity.⁴⁷⁻⁵²

In the Gangetic plains of Bihar, a high percentage of population suffers from arsenic poisoning due to arsenic contamination of groundwater. The long duration exposure to this arsenic has magnified the

toxicity by many folds causing deadly disease like cancer in human beings.⁵³ Recently, the gallbladder cancer disease burden in these areas have increased many times and the situation is catastrophic.⁵⁴ Hence, there is an urgent need to investigate the relationship between arsenic exposure and GBC in the local populace. To date, there has not been any study which shows the linkage between arsenic contamination in groundwater and increased incidence of gallbladder cancer. Therefore, the present study throws new light on the association between gallbladder carcinogenesis and arsenic poisoning in the Gangetic plains of Bihar.

Methods

Ethical Approval: Ethical approval was obtained from the Ethics Committee of the associated institute with (IEC Ref No. 07/RMRI/EC/2019, dated 20/06/2019). All the patients were informed about the research study and written consent were obtained prior to their inclusion in the study.

Study group: Volunteers for this case-control study were selected from those undergoing gallbladder surgeries at the parent cancer institute with the assistance of the institute's Surgical Oncology Department. For the present study, volunteers were divided into confirmed Gallbladder Cancer cases (n=28) and benign Gallbladder or control cases (n=11) after obtaining their consents. Moreover, n=512 gallbladder cancer patients (of institute itself) blood samples were evaluated for arsenic contamination to understand exposure level in the population. The demographic and clinical profiles of volunteers were obtained from the central records of the institute for the baseline information.

Collection of samples: After the surgery, all the biological samples - blood, gallbladder tissue, gallbladder stones, gallbladder bile and hair samples of the volunteers of gallbladder benign as well as the GBC cases were collected. The samples were subsequently stored in -80°C deep freezer prior to the analysis.

Analytical Quality Control: For the analytical quality control, the blanks, procedural blanks, replicates, standards, correlation coefficient and detection limits were maintained for arsenic analysis in the atomic absorption spectrophotometer. The known standard concentration of arsenic was prepared from the standard arsenic stock solution (1000 µg/L) procured from PerkinElmer (CAS# As 7440-38-2; Lot# 20-85 ASX1; PE# N9300102), Singapore. The arsenic detection limit for blood was 0.05 µg/L in the employed method. The cancer patient's tissues, gallstones and bile samples were analysed for the first time in this part of the region, the protocol of National Institute for Occupational Safety and Health (NIOSH, 1994) was utilised.⁵⁵

Analysis of the samples through Atomic Absorption Spectrophotometer:

1. **Analysis of Gallbladder bile and blood samples:** After the collection, all the tissue samples were digested using concentrated HNO₃ (Merck analytical grade 69%) on hot plate under fume-hood and arsenic was estimated as per the protocol of National Institute for Occupational Safety and Health (NIOSH, 1994) by Graphite Furnace Atomic Absorption Spectrophotometer (GF-AAS) (Pinnacle 900T, Perkin Elmer, Singapore) at the studied institute. The blood and gallbladder bile samples were

taken as 0.5ml aliquots in 30 ml conical flask and 5ml HNO₃ was added and left for overnight reaction. The samples were then digested on hotplate at 90°C - 120°C until the sample reached to 3 ml. Then 5ml mixture of HNO₃:HClO₄ (6:1) (HClO₄ - Merck analytical grade 70%) was added to the solution and again re-digested on hotplate until the volume reached to about 2ml. Final volume was adjusted to 10ml with 1% HNO₃. The solutions were filtered through Whatman filter paper no.41 and analysed through GF-AAS.

2. ***Analysis of Gallbladder tissue and Gallstone samples:*** The gallbladder tissue and gallbladder stone samples were taken as 0.5g mass in the conical flask and 5ml of Conc. HNO₃ was then added to the flask and left for overnight reaction. The samples were digested next day on hotplate at 60°C for 2 hours. After that, the samples were cooled and then added with 2ml of HClO₄ and then re-digested at 90°C - 120°C for 5-10 minutes until the white fume of HClO₄ is emitted. The solution was cooled and diluted with 15ml with deionized water and filtered with Whatman filter paper no.41 and analysed by GF-AAS.
3. ***Analysis of hair samples:*** For hair samples analysis, 0.120 g of hair samples were weighed and washed in 15ml of 0.1% SDS solution (Sigma- Aldrich) and sonicated for 10 minutes. The solution was decanted and washed with ultrapure distilled water. Then the hair samples, were placed in 15ml of high-grade acetone solution and sonicated for 10 minutes. The solution was decanted and rinsed with ultrapure distilled water three times. The hair samples were dried at 40°C for 5 minutes in the oven and then 10ml of HNO₃ was added and covered with watch glass and digested on hotplate 90°C - 120°C. When the solution was reduced to 3ml, 1ml of H₂O₂ was added and it was re-digested at the previous temperature until the bubbles came out and the solution was reduced to 2.5ml. Then the heating was stopped, and the solution was rinsed with 1% HNO₃ and adjusted to a final volume of 10ml with ultrapure distilled water. The sample was then filtered through Whatman filter paper no.41 and analysed through GF-AAS.

Molecular Docking analysis of Arsenic compounds with haemoglobin, cysteine and taurine:

1. **Retrieval and preparation of target protein and arsenic compounds:** The 3D- Crystal structure of human haemoglobin (PDB ID: 4HHB) was downloaded from Protein Data Bank (<https://www.rcsb.org/structure/4HHB>) in pdb format.⁵⁶ Human haemoglobin consists of 4 subunits (2 alpha subunit, chain A and chain C; 2 beta subunits, chain B and chain D). The structure was prepared for docking in Chimera X 1.1.⁵⁷ Haemoglobin alpha subunit chain A and beta subunit chain B was used for docking in separate experiments. Arsenous acid (Trihydroxy-arsenite (III), As (OH)₃, PubChem ID 545) 2-D structure was converted to 3D coordinates by MolView web based open-source application and was downloaded as mol file. The ligand molecule was also prepared in Chimera X 1.1 and saved as pdb. The 3D structure of Cysteine (PubChem CID: 5862) and taurine (PubChem CID: 1123) was downloaded from PubChem database as sdf file. These two molecules were also prepared and saved as pdb for further study.

2. **Docking analysis:** Docking was performed by HDock webserver.⁵⁸ The HDock server is to predict the binding complexes between two molecules using a hybrid docking strategy. Firstly, docking was performed between chain A of human haemoglobin and arsenous acid. Haemoglobin was uploaded as receptor and arsenous acid served as ligand molecule. In second experiment human haemoglobin chain B served as receptor and docking was performed with arsenous acid ligand molecule. Similarly, cysteine as receptor was docked with ligand arsenous acid as a separate experiment and taurine as receptor was docked with arsenous acid at HDock webserver as another experiment to understand the interacting residues between two molecules. The results were visualised in Chimera X 1.1 and Pymol Viewer.⁵⁹

GIS Mapping: The data of arsenic concentration in blood samples of the GBC patients (n=512) were taken as input in ArcGIS Version 10.5.1 for spatial analysis and visualization. The location data of collected samples of the GBC patients were plotted and extracted as a GIS map layer for visualization. The study was conducted to understand the spatial pattern of the GBC patients geographically in the arsenic exposed area, and the resultant distribution map result is shown in Fig. 10. The arsenic concentration measured in the five biological samples (tissue, stone, bile, hair and blood) of the GBC patients from the arsenic exposed area were also analyzed in the ArcGIS environment. The output of five biological samples data, location of the GBC patients and the exposure rate pattern is shown in Fig. 16. The software used in the map layer generation was ArcGIS Version 10.5.1.

Statistical analysis:

Data were analysed with statistical software (GraphPad Prism 5) and values were expressed as Mean $\pm$ SEM. Differences between the groups were statistically analysed by one-way analysis of variance (ANOVA) followed by the Dunnett's test for comparisons among study groups. The scatter plot graphs were designed using another statistical software SPSS-16.0 utilising the linear regression model as earlier used.⁶⁰

Results

In the present study, all the collected samples were analysed –

1. **Gender wise patients in gallbladder benign and gallbladder cancer patients:** Out of total 11 gallbladder control (benign) samples, there were 5 male and 6 female patients. Among the confirmed 28 GBCs, there were 8 male and 20 female patients.
2. **Age group of patients in gallbladder benign and gallbladder cancer patients:** Out of the total 11 gallbladder control (benign) cases, the maximum age observed was 68 years, while the minimum was 27 years. Out of 28 confirmed GBCs, the maximum age was 84 years, while the minimum was 35 years (Fig. 1).
3. **Stagewise distribution of gallbladder cancer patients:** Out of 28 confirmed GBCs, there was only one stage-I cancer patient (3.5%), whereas five were stage-II cancer patients (17.85%), two were stage-III

cancer patients (7.14%), and the stage-IV cancer patients were 20, the highest (71.5%) (Fig. 2).

4. **Arsenic concentration in blood samples of gallbladder benign and gallbladder cancer patients:** Out of the total 11 gallbladder benign samples, the maximum arsenic concentration observed was 116µg/L, while minimum was 1.3µg/L. Out of 28 confirmed GBCs cases, the maximum arsenic concentration was 942.8µg/L, while the minimum was 0.901µg/L (Fig. 3).
5. **Arsenic concentration in tissue samples of gallbladder benign and gallbladder cancer patients:** Out of the total 11 gallbladder control (benign) samples, the maximum arsenic concentration observed was 333.6µg/kg, while the minimum was 8.7µg/kg. Out of 28 confirmed GBCs cases, the maximum arsenic concentration was 3627µg/kg, while the minimum was 7.98µg/kg (Fig. 4).
6. **Arsenic concentration in gallstone samples of gallbladder benign and gallbladder cancer patients:** Out of the total 11 gallbladder control (benign) samples, the maximum arsenic concentration observed was 33.4µg/kg, while the minimum was 0.12µg/kg. Out of 28 confirmed GBC patient's the maximum arsenic concentration was 1763µg/kg, while the minimum was 7.98µg/kg (Fig. 5).
7. **Arsenic concentration in bile samples of gallbladder benign and gallbladder cancer patients:** Out of the total 11 gallbladder control (benign) samples, the maximum arsenic concentration observed was 20.8µg/L, while the minimum was 0.17µg/L. Out of 28 confirmed GBCs, the maximum arsenic concentration was 90.4µg/L, while the minimum was 0.03µg/L (Fig. 6).
8. **Arsenic concentration in hair samples of gallbladder benign and gallbladder cancer patients:** Out of the total 11 gallbladder control (benign) samples, the maximum arsenic concentration observed was 680.8µg/kg, while the minimum was 3.30µg/kg. Out of 28 confirmed GBC cases, the maximum arsenic concentration was 4908µg/kg, while the minimum was 11.97µg/kg (Fig. 7).
9. **Correlation Coefficient between gallbladder cancer patient's tissue and bile:** There was a significant positive correlation showing an increasing trend between the arsenic concentrations in the tissue and bile of confirmed GBC patients ($r= 0.14$, $P<0.05$; Fig. 8).
10. **Correlation coefficient between Gallbladder cancer patient's bile and gallstones:** There was also a significant positive correlation and an increasing trend between the arsenic concentrations in bile and gallstones of confirmed GBC cases ($r=0.123$, $P<0.05$, Fig. 9).
11. **Arsenic Concentration in blood samples of n= 512 gallbladder cancer patients:**

The blood arsenic concentration in the n= 512 gallbladder cancer patients showed significant raised levels in n=274 (53.6%) patients having concentration more than 15µg/L, while n=238 (46.4%) had concentration less than 15µg/L. The maximum arsenic concentration observed was 746.43µg/L. In the study, n= 338 were female GBC patients while n=174 were male GBC patients. The n=485 (94.8%) of the GBC patients were in stage IV disease, while n=27 (5.2%) were in stage I, II and III (**Fig. 10**).

12. **Principal Component Analysis (PCA):** Since none of the single biological media could effectively differentiate the cases and controls, PCA was applied to construct a holistic evaluation index. The

used variables were arsenic concentrations in blood samples of gallbladder benign and gallbladder blood (BLOOD), gallbladder tissue (TISSUE), gallbladder stones (STONE), gallbladder bile (BILE), and hair (HAIR) samples. All the variables were standardized. The two principal components (PCs) were found with more than eigenvalue one: PC1 had 1.775 and PC2 had 1.117. Equations (1) and (2) show the formulas used to calculate the PC1 and PC2 scores, respectively, where the coefficients are based on the estimated eigenvector information. **(Fig. 11)** shows the scatter plot of the whole 39 samples' scores in the planar of PC1-PC2.

$$\begin{aligned} \text{PC1 score} = & 0.236 \times \text{BLOOD} + 0.474 \times \text{TISSUE} + 0.666 \times \text{BILE} \\ & + 0.417 \times \text{STONE} + 0.321 \times \text{HAIR} \end{aligned} \quad (1)$$

$$\begin{aligned} \text{PC2 score} = & -0.630 \times \text{BLOOD} + -0.297 \times \text{TISSUE} + 0.160 \times \text{BILE} \\ & -0.096 \times \text{STONE} + 0.693 \times \text{HAIR} \end{aligned} \quad (2)$$

Fig. 11a) demonstrated that all the control subjects had negative scores for PC1. Moreover, according to Fig. 11b), the plots with positive PC1 scores are all from the stage 4 cases. Thus, 13 out of 20 patients from stage 4 were differentiated from other subjects by the PC1 scores, including the controls.

13. Proposed Pathway of Gallbladder carcinogenesis:

14. Molecular Docking: Association between arsenic and its conjugation with sulfhydryl group compounds causing carcinogenesis in Gallbladder Cancer

Interaction study between receptor and ligand molecules: Protein ligand interaction between human hemoglobin chain A and arsenous acid resulted in 7 models where each model represents arsenic interactions at 7 different positions with the protein residues. One of the interacting modes is represented in Fig. 13 where O1 residue of arsenous acid interacts with Cys 104 and SER 35, O3 interacts with Cys 104 and His 103 and O4 residue interacts with SER 35 residues of human hemoglobin alpha subunit chain A. Similarly, docking of hemoglobin chain B with arsenous acid resulted in 12 binding modes for their interactions. Fig. 14 represents one of the interaction modes where Cys 93 and His 146 residues of hemoglobin protein interacts with O2 residue of Arsenous acid. Interaction analysis between cysteine and arsenous acids also resulted in several binding modes. Fig. 15A represents interaction between cysteine and arsenic compound where sulfhydryl group of cysteine is forming hydrogen bonds with O2 and O4 residues of arsenous acid. Interestingly, sulfhydryl group of taurine also interact with arsenic compound (Fig. 15B).

Geological Aspect:

In 18 out of 38 districts in Bihar, have been affected by arsenic contamination in groundwater. Major source of arsenic for human consumption is through arsenic contaminated water.⁶¹ The predominance of gall bladder cases is clearly brought out in the arsenic affected districts of Bihar (**Fig. 16**). The source of arsenic is primarily geogenic as it is acquiring endemic proportions and affecting a large geographical area. The rivers originating from extra peninsular region contain sediments with high arsenic content. The rivers carry these sediments which under favourable conditions releases arsenic in groundwater. The residency time, litho framework of the recharge vis a vis discharge area, age of the water in the affected aquifer and climatic conditions also have a role to play in enhanced contamination. The secondary enrichment in favourable conditions of oxic and anoxic waters supplemented by the geomorphological components also play a decisive role in increased contamination of aquifers at various depth levels. Arsenic contaminated aquifers are mainly present in newer alluvium (Holocene age) which is also supported by a model which defines the primary and secondary source.

Discussion

Arsenic poisoning through drinking water and irrigation water in the recent times has caused serious health hazards in the population worldwide. Prolonged exposure to arsenic leads to many health outcomes. The diseases caused by this long-term arsenic exposure include skin manifestations, hypertension, ischemia, general body weakness, vascular disorders, auto-immune disorders (diabetes, arthritis, leukoderma), severe arteriosclerosis, neuropathies and many types of cancer.^{52,60,62} In most of the cases if these symptoms are ignored, arsenic poisoning may lead to many types of cancer. One of highly correlated health outcome of arsenic poisoning is gallbladder cancer. In recent studies gallbladder cancer has been diagnosed in many patients coming from the arsenic hotspots of Gangetic plains of Bihar.⁶³ However, the etiology of aforementioned diseases leading to carcinogenesis has not been studied extensively. The exposure rate is so much high that one or more members in each exposed household exhibit arsenicosis symptoms.⁶⁴⁻⁶⁶

Arsenic has been classified as a class I human carcinogen by International Agency of Research on Cancer (IARC), which denotes that arsenic possesses the property of not only changing the configuration of the cellular activity but also modifying the gene functions at the genome level in human beings. In the recent studies, skin and several types of internal cancers such as bladder, kidney, liver, prostate and lung have been associated with arsenic ingestion.^{52,67-70} Arsenic usually affects the cellular activities mainly by two pathways – arsenic induced oxidative stress and also through epigenetic changes. The former is mediated by the biotransformation of arsenic leading to the cellular damage through the production of reactive oxygen species (ROS). The ROS typically consists of superoxide anion, hydrogen peroxide, and hydroxyl radical, which can damage the DNA causing chromosomal aberrations. Enhanced arsenic induced production of ROS by the cells has been linked to the carcinogenicity of arsenic.^{32,71-76} The other suggested mechanism of arsenic carcinogenicity is the epigenetic changes which occur due to the changes in DNA methylation, histone modification and microRNAs. The hypermethylation of aberrant DNA promoter is strongly linked with the silencing of the tumour suppressor genes such as gene *p53*. The

gene *p53* is known to be the guardian of the genome, and silencing of this important gene can lead to the transformation of a normal cell into a cancer cell.⁷⁷⁻⁸⁰ The modification of histone proteins by histone acylation is also known to be the cause of arsenic carcinogenicity. Arsenic metabolites have been shown to modulate the normal histone patterns in the cells.⁸¹⁻⁸² The microRNAs are small noncoding RNAs that inhibit the expression of the genes, and they can also induce carcinogenicity by deregulating the normal functions of the cell. The chronic exposure of As^{III} has been suggested to induce malignant transformation.⁸³⁻⁸⁶ Gallbladder cancer in recent time has increased many folds in the Gangetic belt of India. The specific reasons for this increase have not been established yet, but few studies indicated the toxic metals or metalloids and pesticides as the causative agents.⁸⁷⁻⁹²

In the present study, consistently very high arsenic concentrations were recorded in the blood, gallbladder tissue, gallstones, gallbladder bile and the hair samples of gallbladder cancer patients. The comparison of the control gallbladder (benign subjects; n=11) and confirmed gallbladder cancer patients (n=28) showed novel and significant findings. The study also confirms that even the benign group of patients had high arsenic concentration in the blood and tissues, and thus are at the higher risk of converting into a malignant case in the future. There was significantly high arsenic contamination observed in the gallbladder tissue in the benign patients, with maximum concentration of 333.6µg/kg, while in the GBC patient the observed maximum concentration was 3627µg/kg. In gallstones, the maximum arsenic concentration in benign group was 33.42µg/kg, while in the malignant group the maximum contamination was as high as 1763µg/kg. In the gallbladder bile, the maximum arsenic contamination in benign patients was 20.82 µg/L, while the maximum level was 90.39µg/L in gallbladder cancer patients. In the biological markers such as the blood and hair, the maximum arsenic contamination observed in benign patient was 115.7µg/L and 680.8µg/kg, respectively, while the arsenic levels in the blood and hair of gallbladder cancer patients were 942.8 µg/kg and 4908 µg/kg, respectively. There has been no benchmark range setup for the arsenic contamination in gall bladder tissue, bile, stones and blood, but for hair samples the normal levels of arsenic contamination in the unexposed human populaces ranges between 20-200 µg/kg.⁹³ The correlation coefficients between the arsenic levels in tissues and bile, as well as in bile and gallstones in GBC patients were highly significant (r= 0.14 and r=0.123, respectively). Moreover, the n=512 gallbladder cancer patient's blood samples study revealed very significant arsenic concentration in the population of Bihar with maximum arsenic concentration as 746.43µg/L. Out of n=512, n=350 blood samples had relatively had arsenic concentration more than 2µg/L. This means that 68% of the studied population had significant arsenic concentration in their blood. Most of the patients were from the Gangetic plain region in comparison to the non-Gangetic plain area. This positive trend indicates a strong association between arsenic contamination and the observed carcinogenesis.

No signalling pathways have yet found linking arsenic accumulation in the gallbladder tissue or gallstones or gallbladder bile to carcinogenicity. Nonetheless, the present study provides strong circumstantial evidence associating arsenic accumulation with the progression of the gallbladder carcinogenesis. Arsenic in the Gangetic plains of Bihar is also found in the form of arsenopyrite (FeAsS).⁹⁴ It has very high affinity to bind with these two elements, hence it easily binds with endogenous

sulfhydryl compounds including metallothioneins. Arsenic reaches the human blood after the gastrointestinal absorption and may cause gallbladder carcinogenesis in two ways. First, it is hypothesized that, arsenic binds with RBC since haemoglobin in RBC contains the iron, and then after the completion of RBC lifespan (120 days) reaches liver via spleen.⁹⁵⁻⁹⁷ In liver, arsenic binds with cysteine residues which compounds with sulfhydryl group and accumulates further.⁹⁸⁻¹⁰² Subsequently, arsenic binds with the next sulfhydryl group containing compound taurine.¹⁰³⁻¹¹⁰ Taurine is the key factor which controls the formation of the gallstones, however arsenic likely suppresses its function by lowering its levels. This initiates the formation of gallstones with sludge in the gallbladder due to the precipitation of the cholesterol.¹¹¹⁻¹¹² The taurine has the conjugation with the chenodeoxycholic acid (CDCA), that is why high arsenic contamination was observed in the bile of the GBC patients. Gallstones in due course of time causes inflammation in the inner wall of the gallbladder tissue, which after mutations transforms into a cancerous tissue.¹¹² Secondly, it is also hypothesized that arsenic in the gallbladder elicits rigorous production of ROS, leading to frequent mutations in the genes p53, p21, p16 and KRAS. Such mutations in turn induces inflammation in the epithelial cells of gallbladder causing conversion of normal cells to dysplasia, which in the long run leads to gallbladder carcinoma.^{111,113-130}

Molecular Docking studies of arsenic compounds with haemoglobin alpha chain and beta chain have revealed the efficient binding of arsenic compounds with cysteine residues. Arsenous acid was able to bind with Cys 104 residue of hemoglobin alpha chain globin chain A and also binds with the Cys 93 residue of haemoglobin beta chain globin chain B. Other important residues which can be involved in arsenic toxicity to haemoglobin are His, Ser which are efficiently involved in the interaction with arsenic. The toxicity of trivalent arsenicals likely to occur through the interaction of trivalent arsenic species with sulfhydryl groups in proteins. Trivalent arsenicals have high affinity for sulfhydryl groups and can bind to reduced cysteines in peptides and proteins. Several studies have reported the binding of trivalent arsenicals to the cysteine residues of proteins such as iAsIII binds at three cysteine residues, Cys32, Cys34 and Cys 37 of ArsR repressor protein of *E. coli*. Cys156 and Cys206 of Arsenic methyltransferase (AS3MT) of human are the active site for arsenic binding whereas Cys72, Cys174 and Cys224 of AS3MT of thermophilic eukaryotic red algae *Cyanidioschyzon merolae* are believed to be involved in arsenic binding. Hence, the present study also strongly indicates strong association between the chronic arsenic contamination in the Gangetic plains of the Bihar (India) and gallbladder carcinogenesis.

A previous significant study also reported association between the arsenic exposure and gallbladder cancer incidences in the populations of India and Japan. However, the study was conducted using a very small number of gallbladder cancer patients (8 Indian and 5 Japanese GBC cases), and therefore a strong correlation could not be made. Moreover, they also suggested association of gallbladder carcinogenesis with the other heavy metals like chromium, cadmium, lead, zinc and mercury.¹³¹

Similarly, in Chile (South America), the annual cancer incidences are approximately, 35,000 new cases per year and the cancer burden in the population in recent times has increased many folds similar to India.¹³²⁻¹³⁴ The gallbladder cancer incidences in Chile have a higher ratio in comparison to the average

data on a global scale.¹³⁵⁻¹³⁶ Unfortunately, Chile also reports high arsenic concentration in the groundwater with concentrations as high as about 5,000µg/L.¹³⁷ Not much is known about the association between arsenic exposure and increased gallbladder cancer cases in Chile.¹³⁸⁻¹³⁹ Recent studies, reported that if the exposed subjects are consuming arsenic contaminated water for long duration may cause risk of gallbladder cancer.¹⁴⁰⁻¹⁴¹

The PCA results demonstrated that the internal arsenic concentrations could not characterize all the severe gallbladder patients, but all the highly arsenic-loaded subjects were the severe gallbladder patients. This suggests the association between arsenic intake and gallbladder cancer. The high internal arsenic concentration may cause accelerating the progress of gallbladder cancer. Another suggestion was that a single biological medium might not be sufficient to understand the real affection of the internal arsenic concentrations: e.g., some patients had high arsenic concentrations in gallbladder tissues, but hair samples showed low concentrations and vice versa. The way how arsenic is metabolized and accumulated in bodies may differ depending on the individuals.

Overall, it can be concluded from the present study that gallbladder cancer is prevalent in the Gangetic plains of Bihar, where the arsenic contamination in the exposed population is very high increasing the disease burden of the state. The significantly high arsenic concentration observed in the gallbladder tissue, gallbladder stone, bile, blood and hair samples in GBC patients strongly indicates the linkage between high chronic exposure to arsenic and gallbladder carcinogenesis. The present study also indicates that the benign gallbladder subjects if left untreated for years are susceptible to gallbladder carcinogenesis with sustained arsenic exposure. However, the other confounding factors can also add the disease burden of GBC manifolds. Hence, there is an urgent need to control the disease burden by developing policies and guidelines that will alleviate arsenic exposure to the impacted populations.

Declarations

Data availability

All the data materials have been shared to the journal which on request can be obtained from the journal.

Competing interests

The authors declare that they have no conflict of interests.

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Figures

Figure 1

Age group of the patients in control versus confirm gallbladder cancer patients (ANOVA- Dunnett's Test, $P<0.05$).

Figure 2

Stagewise distribution of gallbladder cancer patients (ANOVA- Dunnett's Test, $P<0.05$).

Figure 3

Arsenic concentration in blood samples of patients in control versus confirmed gallbladder cancer patients (ANOVA- Dunnett's Test, $P < 0.05$).

Figure 4

Arsenic concentration in tissue samples of patients in control versus confirmed gallbladder cancer patients (ANOVA- Dunnett's Test, $P < 0.05$).

Figure 5

Arsenic Concentration in gallstone samples of patients in control versus confirmed gallbladder cancer patients (ANOVA- Dunnett's Test, $P<0.05$).

Figure 6

Arsenic Concentration in bile samples of patients in control versus confirmed gallbladder cancer patients (ANOVA- Dunnett's Test, $P<0.05$).

Figure 7

Arsenic Concentration in hair samples of patients in control versus confirmed gallbladder cancer patients (ANOVA- Dunnett's Test, $P<0.05$).

Figure 8

The scatterplot graph of gallbladder cancer patient's tissue and bile ($r= 0.14$ & $P<0.05$).

Figure 9

The scatterplot graph of gallbladder cancer patient's bile and gallstones ($r= 0.123$ & $P<0.05$).

Figure 10

Fig. showing distribution of GBC cancer patients ($n=512$) (Arc-Gis- 10.5.1)

Figure 11

The scatterplot graph on the PC1-PC2 planar: a) Case-Control, b) Case Stage-Control.

GALL BLADDER CARCINOGENESIS

ARSENIC EXPOSURE TO HUMANS

Figure 12

Schematic diagram showing the etiology of Gallbladder carcinogenesis

Figure 13

3D structure of Human Haemoglobin (PDB ID: 4HHB, Alpha subunit, Chain A) bound with As III (Arsenous acid, PubChem ID: 545) at Cys 104 residue after molecular docking. A: Surface model of 4HHB showing arsenic bound with Cys 104, B: Enlarged view of the interaction showing interaction of Arsenic with cysteine sulfhydryl group (yellow colour), C: Hydrophilic and Hydrophobic interaction of the As III (arsenous acid) bound with chain A of alpha subunit of human haemoglobin.

Figure 14

3D structure of Human Haemoglobin (PDB ID: 4HHB, Beta subunit, chain B) bound with As III (Arsenous acid, PubChem ID: 545) at Cys 93 residue after molecular docking. A: Surface model of 4HHB showing arsenic bound with Cys 93, B: Enlarged view of the interaction showing interaction of Arsenic with cysteine sulfhydryl group (yellow colour), C: Hydrophilic and Hydrophobic interaction of the As III (arsenous acid) bound with chain B of beta subunit of human haemoglobin.

Figure 15

A- Cysteine (PubChem CID: 5862) sulfhydryl group bound to As III (Arsenous acid, PubChem CID:545) after molecular docking, **B:** Taurine (PubChem CID: 1123) sulfhydryl group binds with As III (Arsenous acid, PubChem ID: 545).

Figure 16

Map showing studied Gallbladder cancer patients with arsenic concentration in different biological samples with geomapping (Arc-Gis-10.5.1)