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Original Research Article

The Cell Proliferation Index and Clinicopathological Features in Arsenic Related Bladder Cancer of West Bengal: A Single Institute Case Control Study

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Abstract

Background: Bladder cancer is one of the most common urological malignancies. Bladder cancer is caused by genetic abnormalities and external risk factors, including carcinogen exposure, nutritional factors, fluid intake, alcohol, inflammation, infection, chemotherapy, radiation and possibly artificial sweeteners. Arsenic consumption as a causative factor in bladder carcinoma is studied here.

Aims: To study whether arsenic levels in serum, urine and bladder tissue are higher in carcinoma bladder patients and the relationship between blood arsenic levels and the grade of bladder cancer.

Methods: This cross-sectional observational study was conducted at coming to Ramakrishna Mission Seva Pratishthan Hospital between June 2014 to June 2016. 102 patients who underwent TURBT were assessed for blood arsenic levels and tumour arsenic levels. 51 of them were already diagnosed with bladder carcinoma and 51 were controls.

Results: Significantly ($p<0.001$) higher percentage of bladder tumour with advanced clinical stage was found among arsenic positive cases than that of arsenic negative cases. A significantly higher level of expression of PCNA was recorded in tumour samples compared to the control. A higher frequency of expression of PCNA in arsenic-positive samples compared to that of arsenic-negative samples in both cases and controls was found ($p<0.001$).

Conclusions: Arsenic endemicity is associated with a higher incidence of high-grade cancers in comparison to low grade bladder cancers. Detecting arsenic positivity in bladder cancers may help in changing our way of managing of arsenic-positive bladder cancers, i.e., arsenic-related bladder cancers do demand more aggressive therapeutic intervention and more intensive follow-up.

Keywords: Bladder Carcinoma, Arsenic, PCNA, Blood Arsenic Levels.

Introduction

Bladder cancer is one of the most common urological malignancies. As per the Indian Cancer Registry data it is the ninth most common cancer, accounting for 3.9% of all cancer cases. [1] The mean age at diagnosis is 69 years old for males and 71 years old for females. [2] Bladder cancer is caused by genetic abnormalities and external risk factors, including carcinogen exposure, nutritional factors, fluid intake, alcohol, inflammation, infection, chemotherapy, radiation and possibly artificial sweeteners. Arsenic consumption is one such risk factor. The US (United States) EPA (Environmental Protection Agency) finalized a proposed standard for arsenic in drinking water that lowered the MCL (Maximum Concentration Level) from 50 parts per billion (ppb) to 10 ppb. [3] Our study focussed on the possible role of arsenic toxicity in the development of bladder cancer. Consumption of arsenic in drinking water was the main focus of this work; however, other exposure pathways are also important.

Aims and Objectives

To study whether arsenic levels in serum, urine, and bladder tissue are higher in carcinoma bladder patients and if patients with bladder carcinoma and high arsenic levels are prone to a higher grade of cancer, a higher stage of cancer, and more frequent recurrences.

Methods and Materials

The study was conducted on patients attending to Ramakrishna Mission Seva Pratishthan Hospital between June 2014 and June 2016. Arsenic estimation was done in the Department of Biological Science, Presidency University (Kolkata) and PCNA estimation was done in the Department of Oncogene Regulation, CNCRI.

All patients coming from different parts of West Bengal for the management of bladder tumours formed the study group. Patients who underwent cystoscopy for reasons not related to bladder tumour were included in the control group.

This was a case-control study, patients with bladder cancer constituted the cases, and patients undergoing cystoscopy for causes other than bladder tumours constituted the controls.

As per the study of bladder tumour by Chow NH et al., the proportion of grade-3 tumour among arsenic-exposed patients and among non-arsenic-exposed patients was 45.65% (n = 46) and 20.31% (n = 64), respectively.

The formula for calculating the sample size in a case-control study was:

$n = (Z_{\alpha/2}+Z_{ß})^2 * (p_1(1- p_1)+ p_2(1- p_2))/(p_1- p_2)$,

Where $Z_{\alpha/2}$ is the critical value of the normal distribution at α/2 (e.g., for a confidence level of 95%, α is 0.05 and the critical value is 1.96), $Z_{ß}$ is the critical value of the normal distribution at β (e.g., for a power of 80%, β is 0.2 and the critical value is 0.84), and p_1 and p_2 are the expected sample proportions of the two groups.

Thus, there is a need for 51 study subjects per group with 80% power at a 95% confidence level. The number of patients in each group was in the ratio of 1:1. Thus, the required sample size for the study was 102.

The duration of our study was two years (June 2014 to June 2016); bladder cancer patients were followed up by cytology and check cystoscopies once in every three months.

All patients coming to our hospital with bladder cancer and giving consent to being included in our study and who were also ready for follow-up were included in our study. Patients undergoing cystoscopy for reasons other than bladder cancer and giving consent for a bladder biopsy were enrolled as controls.

Patients coming from outside West Bengal or not willing to give informed consent were excluded from our study.

The address (special focus on whether from an arsenic endemic zone or not), personal and/or family history of participants were recorded in a well-prepared questionnaire, including smoking and drinking habits, arsenic contamination in drinking water, occupational exposure to carcinogens, family history of cancer, etc. All patients with haematuria and those with risk factors for bladder tumours as well as those who were planned for cystoscopies, were subjected to CECT KUB and urine cytology. Intraoperatively, a bimanual examination under anesthesia was done in each patient before cystoscopy and tumour resection. This was repeated after tumour resection to look for residual palpable mass to clinically assess the stage of tumour. Cystoscopy findings were noted regarding gross appearance. Histopathological staging and grading of all tumour patients were noted.

Patients undergoing surgery for Ca urinary bladder (TURBT or cystectomy) at our institute between June 2014 and June 2016 were enrolled after informed consent was obtained from them.

Freshly operated bladder tumour tissues from the affected patients, along with 5 ml of blood and a morning urine sample, were collected. After informed consent, similar samples were collected from patients undergoing cystoscopy for other causes other than malignancy as a control group. To confirm that the patients in the second group do not have bladder malignancy, a part of the bladder tissue obtained was sent for histopathological examination, and if this came out to be positive for malignancy, they were shifted to the first group.

Bladder tissue was divided into three parts: the first part went for histopathological examination in a formalin vial; the second part also went in a formalin vial for immunohistochemical analysis of PCNA for proliferation index determination; and the third part was sent for arsenic estimation in 'Eppendorf'.

We determine arsenic (III) at the ppb level from the test samples. At first, tumour tissue was subjected to acid digestion, i.e., 0.5–1 g of semi-air-dried bladder tissue were digested by a 5 ml acid mixture, (1:3 HNO_3:$HCIO_4$) on a temperature-controlled hot plate, and 20 ml of double-distilled water and 1 ml of conc. HCI were added after digestion to make a final volume of 25 ml. The acid digested sample was divided into two parts: one, arsenic level was detected using a silver-bis (thiophene-2-aldehydo) thiocarbohydrazone (S. Palchaudhury et al., 2007) complex (BTATCH) as an absorbent by spectrophotometer; and in another part, arsenic level determination was done by an atomic absorption spectrophotometer with FIAS (Flow Injection Analytical System) assembly for data validation.

Arsenic was detected using a kit-based method according to the manufacturer's protocol (Lamotte, Maryland, USA). Briefly, inorganic As+3 and

As+5 are converted to arsine gas. This reacts with the test strip in a closed container and produces yellow-to-brown colours on the strip. The strip colours are compared to a colour chart to determine the arsenic concentration.

For the purpose of our study, we have defined bladder cancer patients as being arsenic-related if they came from an endemic zone or had high serum, tissue, and urine arsenic levels.

The clinicopathological characteristics of arsenic-related bladder cancers were then compared to non arsenic-related ones. All tumour patients were followed up once in every three months for urine cytology and cystoscopy, as per the recommended follow-up schedule.

Statistical Analysis

Statistical analysis was performed with the help of Epi Info (TM) 3.5.3, which is a trademark of the CDC (Centres for Disease Control and Prevention).

Using this software, basic cross-tabulation and frequency distributions were prepared. The X^2 test was used to test the association between different study variables. The corrected x test was used in cases where any one of the cell frequencies was found to be less than 5 in the bivariate frequency distribution.

A test of proportion (X^2-test) was used to test the significant difference between two proportions. T-test was used to test the significant difference between means.

OR (Odds Ratio) with a 95% CI (Confidence Interval) was calculated to find the risk factors. $P<0.05$ was taken to be statistically significant and confidence intervals was set at 95%; $p<0.05$ was considered statistically significant.

Results

In our study, for the purpose of significance in statistical analysis, at least 51 cases and 51 controls were required; at the end of our study, we have 54 tumour cases and 54 controls. Our calculations, observations, and results are based on 54 cases and 54 controls.

In our study, there were no patients below the age of 40. There was a single patient between 40 and 49 years old; there were 9 patients in their fifties, majority (N = 25; 46%) of the patients were in their sixties; 18 patients (33%) were in their seventies; and there was a single patient above 80 years old.

It was found that of the total 54 cases, only 10 occurred below 60 years of age, and of those, 8 were from arsenic-endemic areas, and one of those 8 cases was only 44 years of age. This occurrence of a higher number of bladder cancers in the younger age group in cases of arsenic positivity is found to be statistically significant.

Out of the 54 cases, there were 50 male patients and 4 female patients. Of the 54 cases, 7 (13%) were of pTa low grade, 23 (42.6%) were of T_1 low grade, 21 (38.9%) were of T_2 high grade, and 3 (0.56%) cases were of T_3 and T_4 stages.

In 54 tumour cases, 45 were of high grade and 9 were of low grade. All the superficial tumours (pTa) were of low grade, and all the T1 and other invasive tumours were of higher grade.

Out of 54 tumours patients, 33 were chronic smokers and 21 were non-smokers, and of the 51 controls, there were 26 chronic smokers and 28 non-smokers.

The difference between smokers and non-smokers among tumour and non-tumour patients was found not to be statistically significant. 31 (57.4%) of our 54 tumour patients are from arsenic-endemic areas of West Bengal, while 19 (37.2%) of our 51 controls were from arsenic-endemic zones. This difference in arsenic background is found to be statistically significant ($p = 0.021$). In our study, a good number of the case and control individuals (cases > controls) were from arsenic-affected districts. Our hospital is in Kolkata, adjacent to South and North 24 Parganas. In accordance with district-wise documentation, the highest prevalence of the disease is in Kolkata, followed by South 24 Parganas, Howrah, North 24 Parganas, etc. However, the majority of the cases were from arsenic-affected blocks, and controls were from arsenic-safe blocks in a district.

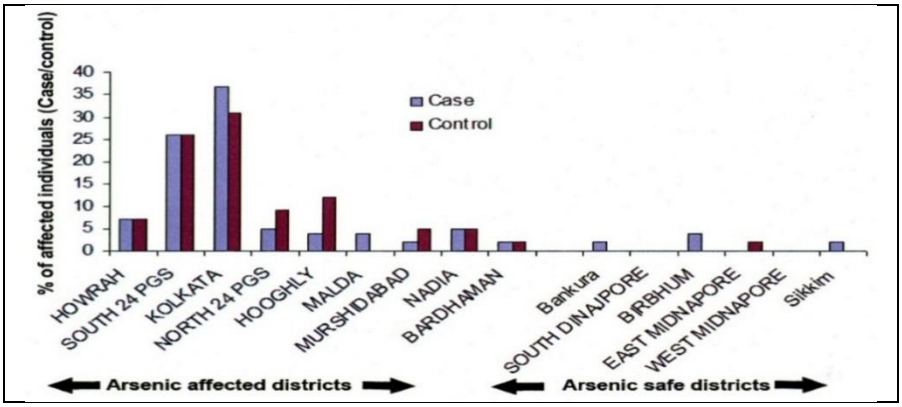

Figure 1: Distribution of Case and Control Individuals in Respective Districts of West Bengal.

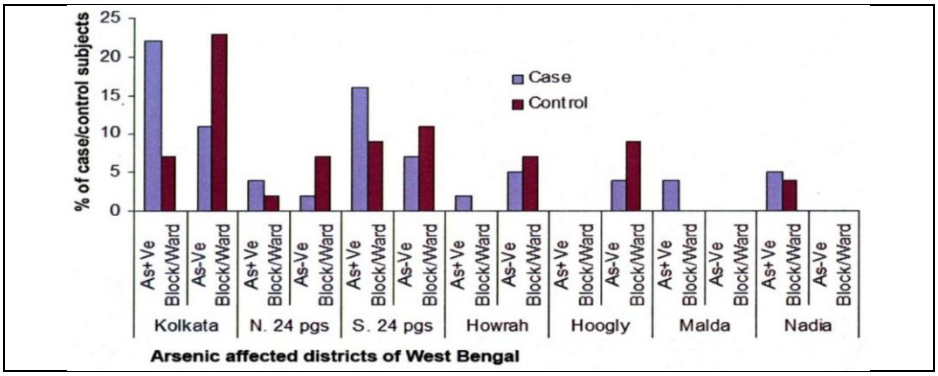

Figure 2: Distribution of Case and Control Subjects in Arsenic Positive and Arsenic Negative Blocks or Wards of Different Districts of West Bengal

Arsenic Level in Tissue, Blood and Urine

In our study, out of the total number of patients, 50 were from arsenic-endemic areas and 58 were from arsenic-safe areas. For the purpose of our study, we have defined bladder cancer patients as being arsenic-related if they came from an endemic zone and if they had high serum, tissue, and urine arsenic levels. Of note, 2 patients in our study were not from an arsenic-endemic zone but had high arsenic levels and were thus taken as having arsenic-positive cancer.

The arsenic estimation in tissue, blood, and urine is expressed in terms of parts per billion (ppb). The mean level of tissue arsenic in patients coming from the arsenic endemic area is 189.18±101.59 ppb, and that of those coming from the arsenic safe area is 51.77±33.84 ppb.

The blood arsenic in patients coming from arsenic endemic areas was 18.32±8.59 ppb, and that of those patients coming from arsenic safe areas was 6.2±3.27 ppb. Urine arsenic of patients coming from arsenic endemic areas is 213.44±100.82 ppb and from arsenic safe areas was 59.7±26.37 ppb.

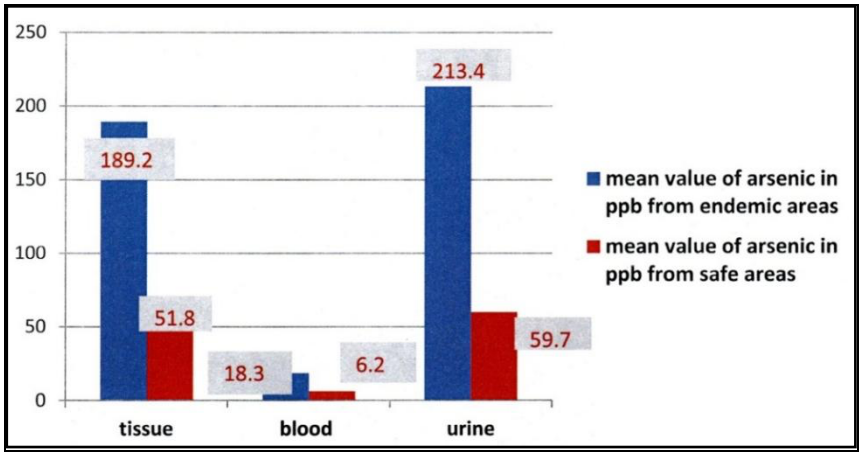

Figure 3: Average Levels Arsenic in Tissue Blood Urine in Patients from Arsenic Endemic and Arsenic Safe Areas

In our study, all the cases and control individuals from the arsenic-safe area showed an average arsenic level of 58 ppb and 55 ppb, respectively, in the urinary bladder, which was therefore considered the permissible tissue arsenic level accordingly. 70% (38/54) of the case and 59% (30/51) of the control were considered to have arsenic-positive bladder tissue, and furthermore, it showed concordance with respect to arsenic status in the respective blood and urine samples.

We did association studies of the tissue arsenic status with different clinicopathological parameters of tumour like clinical stage, histopathological grade, etc. Significantly ($p<0.001$) higher percentage of Bladder tumor with advanced clinical stage was found among arsenic positive cases than that of arsenic negative cases. However, such associations did not remain significant in higher histopathological grades.

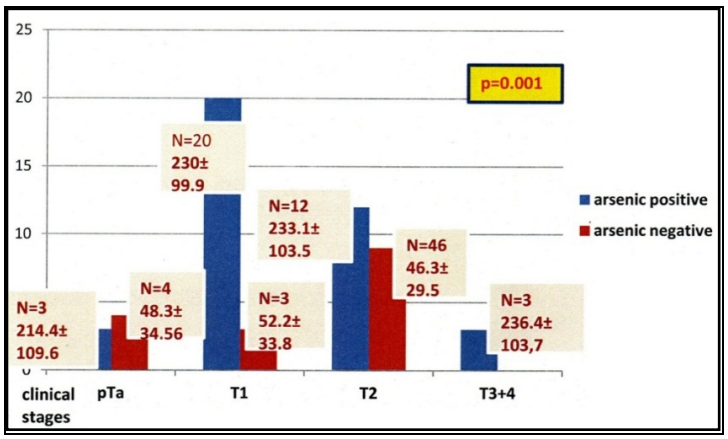

Figure 4: Relationship between Arsenic Positivity and Stage of Tumour

The mean arsenic levels of bladder tissue from cases in endemic and safe areas are 215.6±96.2 ppb and 67.3±31.6 ppb, respectively. The mean arsenic levels in the blood of tumor cases from endemic and non-endemic areas are 19.8±7.9 ppb and 5.7±2.9 ppb, respectively. The mean arsenic levels in the urine of tumor patients from arsenic-affected and safe areas are 228.8±100.3 ppb and 63.5±24.6 ppb, respectively.

In our study, among 45 high-grade bladder cancer cases, 32 (71.11%) were arsenic positive, and of the 9 low-grade cases, 6 (66.67%) were arsenic positive. This relationship between arsenic positivity and grade of bladder cancer was not found to be statistically significant ($p = 0.101$).

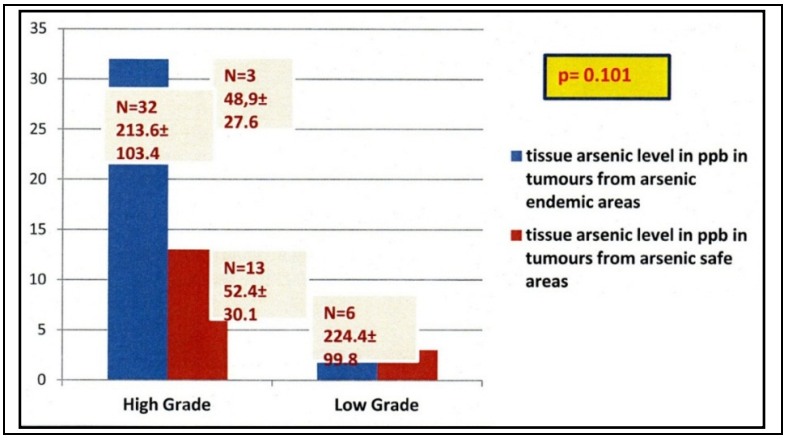

Figure 5: Relationship between Arsenic Positivity and Grades of Tumours

Assessment of Arsenic Level with Proliferation Index in Bladder Carcinoma

In immunohistochemical analysis, expression of PCNA was nuclear in both normal bladder tissue and tumour samples. A significantly higher level of expression was recorded in tumour samples compared to the control. Furthermore, we did an association analysis of the expression level of PCNA with the tissue arsenic status. A significantly higher frequency of expression of PCNA in arsenic-positive samples compared to that of arsenic-negative samples in both cases and controls was found (Tables 1 and 2). Thus, arsenic, in the case of chronic exposure, enhances cell

proliferation, which might contribute significantly to adverse tumour phenotypes.

Table 1: Association of Proliferation Index between Normal and Tumour Samples

	Normal	Tumour	P-Value
High	**21 (39%)**	**37 (68%)**	**0.002***
Normal or Intermediate	**33 (61%)**	**17 (32%)**	

Table 2: Association of Proliferation Index between Arsenic Positive and Negative Tissue Samples of Control and Case Subjects

	Normal (Control)		P-Value	Tumour (Case)		P-Value
	As+	**As-**	**0.005***	**As+**	**As-**	**0.001***
High	17	4		31	6	
Normal or Intermediate	14	19		7	10	
As : Arsenic Status, * p Value significant						

In our follow-up of two years, we had only one recurrence, and this occurred in a 67-year-old lady who was found to be arsenic positive; however, to conclude a statistical significance from such a small number and a shorter follow-up is not possible.

Discussion

Most of the tumor patients in our study are in their seventh and eighth decades of life (seventh>eighth). Parag Gupta et al. have shown a similar finding in their study, with the mean age of presentation of bladder cancer being 60.2 +/- 4.4 years. [4]

In our study, the average age of the tumor patients coming from arsenic-endemic areas was lower than the average age of tumor patients coming from arsenic-safe areas. This difference in the average age of presentation was found to be statistically significant. Arsenic exposure causes bladder cancer at a younger age.

In our study cohort, we found only 13% (N = 7) pTa tumours, while pT_1 and pT_2 tumours constitute 42.6% (N = 23) and 38.9% (N = 21) of cases, while pT_3 and pT_4 tumours constitute 0.56% (N = 3) of cases. Different researchers in different parts of the world have found different prevalences of different stages of bladder cancer (Matthew E. Nielsen et al. [5] and Guido Barbagli et al. [6]).

In our study, 16.67% (N = 9) of cases were of low grade, and 83.33% (N = 45) cases were of high grade. Gregory B. Boustead et al. [7] have found in their case series that 65% of cases were of low grade while 35% were of high grade. 61% (N = 33) of our cases were chronic smokers, while 51% (N = 26) of our controls were chronic smokers, and this difference in the number of smokers in the cases and controls is not found to be statistically significant. So, it appears that smoking is not associated with an increased risk of bladder cancer, while alcoholism is. Smoking has a very strong association with bladder cancer (Maximilian Burger et al. and Neel D. Freedman et al. [8] This discrepancy can be explained as occurring due to the selection bias of our controls. The aim of our study was to detect the association between arsenic and bladder cancer. Arsenic is consumed by both sexes in equal proportion from the environment; as a result, we applied no measures to overcome the bias that might arise while calculating the association of bladder cancer with alcohol and tobacco. The other reason for this discrepancy may be due to our small sample size.

57% (N = 31) of our cases are from arsenic endemic zones, while 37.2% (N = 19) of our controls are from the arsenic endemic zones of West Bengal. This relationship between arsenic endemicity and bladder cancer is found to be statistically significant.

Meta-analysis based on epidemiological studies by Saint Jaques et al. [9] and Christoforidou EP et al. [10] has found a positive relationship between bladder cancer and arsenic exposure.

N-H Chow et al. [11] and Lee E. Moore et al. have found a positive relationship between arsenic positivity and the grade of tumours. On the contrary, Molka Feki-Tonusi et al. [12] and Victor D. Martinez et al. [13] did not find any positive relationship between arsenic positivity and grades of tumour. In our study, 71% (N = 32) of the high-grade cases were arsenic-positive, and 66.67% (N = 6) of the low-grade cases were arsenic-positive. This relationship between grades and arsenic positivity is not found to be statistically significant, which may be because of our small sample size in our study and the overall lower number of low-grade cases in our study. However, it is to be noted that the majority of tumour cases in our study are of high grade, which is much higher than the prevalence of high-grade tumours in the data

available worldwide. Therefore, we can safely conclude that, because of arsenic endemicity, the number of high-grade bladder cancer cases in our study was much higher than the low grade cases in comparison to worldwide data.

The presence of arsenic in both cases and controls is an indicator for public health measures in this regard.

We explored the association of arsenic with clinicopathological parameters of bladder cancer. A significant association was found between tissue arsenic status and the higher clinical stage of bladder cancer.

Similar to us, Chung-Hsin Chen et al. (2009) [14] and Lee E. Moore et al. [15] have found that bladder tumors from arsenic-exposed patients may behave more aggressively in terms of stage and grade. On the contrary, N-H Chow et al. [11] observed no apparent correlation with tumor size, staging, gross configuration, multiplicity, patient gender, or age at diagnosis.

As a probable explanation for the association of arsenic with adverse tumour phenotypes, we analysed the proliferation potential of bladder cancer. The significantly higher proliferation potential of arsenic positive tissue than that of arsenic-negative tissue provided a probable explanation in this respect. Similar to us, Motiwale et al. (2005) [16] also reported the association of sodium arsenate with enhanced PCNA (nuclear protein; indicative of cell proliferation index) expression in mouse skin tumour. Christine Kim et al.[17] have also found that arsenic inhibits the mismatch repair gene in tissues and causes increased expression of PCNA.

Conclusion

Arsenic is a major source of contamination of drinking water in most parts of West Bengal, causing increased exposure for individuals residing in those areas, putting them at increased risk of health-related consequences of arsenic exposure. Arsenic endemicity is associated with a higher incidence of high-grade cancers in comparison to low-grade bladder cancers. Because arsenic exposure is a risk factor for bladder cancer, measures can be implemented at the epidemiological level to prevent arsenic exposure and decrease the incidence of bladder cancer in arsenic-endemic regions of West Bengal. Detecting arsenic positivity in bladder cancers may help in changing our way of managing arsenic-positive bladder cancers, i.e., arsenic-related bladder cancers do demand more aggressive therapeutic intervention and more intensive follow-up.

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