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Environmental and lifestyle factors in epigenetic regulation of bladder cancer: Impact on CALCA and CCNA1 gene methylation

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Requirement for non-invasive biomarkers is emerging, which can decipher lifestyle exposures to epigenetic alterations in bladder cancer due to high recurrence of the disease, and currently used tests like urine cytology with low sensitivity for such tumours. There is a high influence of lifestyle and environmental factors on Urinary bladder cancer (UBC), particularly smoking and chemical exposures. This study evaluates the methylation status of CALCA and CCNA1 genes in UBC patients. It also examines their association with smoking and occupational chemical exposure. A case-control study was conducted with 89 patients with bladder cancer and 80 healthy controls. Methylation-specific PCR (MS-PCR) was used to assess promoter methylation in blood and tissue specimens. Associations of UBC with risk factors, recurrence, and clinicopathological features were studied. The results reflect hypermethylation of the CALCA and CCNA1 genes in UBC cases (66.2% and 53.9%) as compared to controls (1.25% and 2.5%, $P< 0.0001$). In our study, smoking has been correlated with methylation of CALCA ($P= 0.0001$) and CCNA1 ($P= 0.0038$)genes , whereas chemical exposure associated the CCNA1 gene methylation ($P< 0.0005$) with a major link with recurrence. Gene Methylation associations were not found with age, gender, or tumour grade.

These findings point out that environmental factors influence CALCA and CCNA1 methylation in the development of urinary bladder cancer. Additionally, CCNA1 methylation may serve as a biomarker for recurrence, emphasising the importance of epigenetic monitoring in high-risk populations. Taken together, these data support methylation-based, minimally invasive risk assessment in high-risk groups (smokers and occupationally exposed workers) and recurrence surveillance to complement cystoscopy in routine follow-up.

Keywords: Epigenetic surveillance, Methylation-specific PCR, Occupational exposure, Recurrence biomarker, Tobacco smoking, Urothelial carcinoma

One of the most common Malignancies amongst the global cancer incidence is Urinary Bladder cancer (UBC), which is ranked tenth. Around 18926 cases of UBC are being diagnosed every year with approximately 10,000 deaths annually in India[1-2]. UBC is prevalent more in males as compared to females, which is attributed to more exposure to environmental carcinogens, occupation-linked hazards, and smoking habits[3]. Although technology is advancing to detect disease at an early stage, there is still a high rate of recurrence (50–70%) and tumour progression (10–20%) along with highly complex clinical challenges[4-5]. Current diagnostic methods, such as invasive cystoscopy and non-invasive urine cytology, though slightly more effective, are costly and still have limited sensitivity (38%) for low-grade tumours[6].

The environmental carcinogens resulting from changes in lifestyle habits contribute largely to bladder cancer pathogenesis, with up to 80% of cases linked to it. Smoking tobacco is one of the most significant factors, as it alone increases the risk of UBC by 3-5 times, as it exposes one to aromatic amines, polycyclic hydrocarbons, and nitrosamines[7]. Exposure to chemicals used in industries manufacturing dyes, leather, petroleum, and rubber has been found to be a valid contributor to such oncology conditions[8]. These chemicals induce epigenetic modifications, such as DNA methylation, that result in gene silencing and ultimately disrupt cellular functions[9].

Epigenetics is a promising field in cancer research, with DNA methylation being one of the most common amongst all the modifications. This involves

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adding methyl groups to cytosine residues at CpG islands, leading to transcriptional repression. Aberrant hypermethylation of tumor suppressor genes is a critical event in bladder carcinogenesis, making methylation-based biomarkers highly attractive for non-invasive cancer detection and risk assessment[2].

Among the various genes associated with bladder cancer, CALCA (*Calcitonin Alpha*) and CCNA1 (*Cyclin A1*) have emerged as promising epigenetic biomarkers[10]. CALCA gene helps in calcium homeostasis and immune modulation. The enhanced methylation causes silencing of the gene, resulting in tumour initiation and evasion of the immune system[9]. On the other side, CCNA1gene is a regulator of the cell cycle and apoptosis which is found frequently hypermethylated in high-grade UBC cases, causing uncontrolled cell proliferation[11].

In this study, the methylation status of CALCA and CCNA1 genes has been measured qualitatively in urothelial cancer cases by mapping to their association with smoking, occupational chemical exposure, and recurrence risk. In this study,the aim is to map the methylation status of genes in high-risk populations by comparing blood and urine specimens from cases and controls to establish their role as epigenetic markers. Epigenetic modifications, such as DNA methylation, are studied, and these alterations correlate with tumour recurrence, histopathological grade, and demographic factors. The findings could weave the pathway for the development of non-invasive methods to facilitate early diagnosis and prognosis in UBC, ultimately decreasing reliance on invasive procedures such as cystoscopy[5].

Materials and Methods

Study Design and Population

It is a case-control study evaluating the methylation status of the CALCA and CCNA1 genes in UBC patients, with the aim of associating it with environmental and lifestyle factors. The study was conducted at a tertiary care centre following ethical approval obtained from the institutional ethics committee. Written consent was taken from all participants (Fig. 1).

Inclusion and Exclusion Criteria

Inclusion Criteria:

- Histopathologically confirmed urothelial bladder cancer (UBC) cases.
- Patients diagnosed with bladder cancer of any stage and grade.
- Age and sex matched healthy individuals with no history of malignancy.
- Individuals willing to provide informed consent.

Exclusion Criteria:

- Patients with a history of cancer treatment (chemotherapy or radiotherapy).
- Individuals with other genitourinary malignancies.
- Patients having autoimmune diseases or chronic inflammatory conditions affecting the bladder.
- Patients with a history of cysts or benign tumors of the urinary bladder.

Sample Collection: Biological samples were collected from 89 bladder cancer patients and 80 healthy controls.

Blood Samples: Whole blood (5 mL) was collected in EDTA vacuum tubes prior to any surgical intervention.

Tissue Samples: Biopsy specimens were obtained during transurethral resection of bladder tumors (TURBT).Samples were preserved in All protect microcentrifuge tubes for DNA extraction.

DNA Extraction and Bisulfite Conversion

DNA was extracted from blood as well as tissue specimen using the QIAamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's protocol.The quality & concentration of the extracted DNA had been verified using NanoDrop spectrophotometry (Thermo Fisher Scientific, USA).Bisulfite conversion of extracted DNA was performed using the EpiTect Bisulfite Kit (Qiagen, Germany) to enable methylation-specific PCR (MS-PCR).

Methylation-Specific PCR (MS-PCR)

The methylation status of CALCA and CCNA1 genes was assessed using methylation-specific PCR

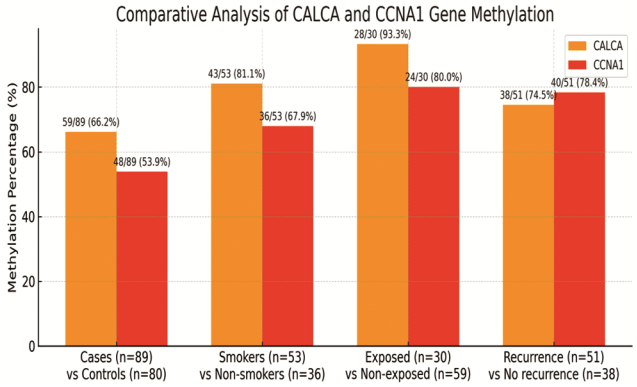

Fig. 1 — Comparative methylation rates of CALCA and CCNA1 across cases and controls, stratified by exposure and recurrence. Cases n=89; Controls n=80; Smokers n=53; Non-smokers n=36; Occupationally exposed n=30; Non-exposed n=59; Recurrence n=51; No recurrence n=38. Bars show methylation percentages

(MS-PCR).Specific primers for methylated and unmethylated sequences of CALCA and CCNA1 genes were designed using MethPrimer. Primer sequences were validated based on previously established guidelines and methodologies[12-13] as shown in (Table 1). The PCR amplification was carried out in a Thermal Cycler (Applied Biosystems, USA) under the following conditions:

- Initial denaturation: 95°C for 10 min
- 40 cycles of denaturation (95°C for 30 sec), annealing (58°C for 30 sec), and extension (72°C for 30 sec)
- Further extension at 72°C for 10 min

The PCR products were subjected to 2% agarose gel electrophoresis, and bands were visualized under UV transillumination (Bio-Rad, USA).

Histopathological Grading and Staging

Histopathological evaluation of tumor tissue was performed by an expert pathologist using Hematoxylin and Eosin (H&E) staining. Tumors were classified based on histological grade and TNM staging[14-15].

Statistical Analysis

Data analysis was performed using R Studio. Associations between methylation status and clinical/environmental factors were assessed using:

- Chi-square test for categorical variables
- Logistic regression to determine odds ratios (OR) with 95% confidence intervals (CI)
- A p-value < 0.05 was considered statistically significant.

Results

A total of 89 bladder cancer patients and 80 healthy controls were considered in the study. The mean age of patients was 60.56 ± 5.85 years, ranging from 32 to 85 years, while controls had a mean age of 45.37 ± 10.75 years ($P< 0.05$). The majority of bladder cancer cases (68.5%) were above 55 years of age, whereas a larger proportion of controls (58.75%) were below 55 years. Gender distribution showed a significant male predominance (80 males, 9 females in cases vs. 65 males, 15 females in controls). The association of male gender with bladder cancer was statistically significant ($P< 0.01$). The methylation status of CALCA and CCNA1 genes was analyzed using Methylation-Specific PCR (MS-PCR) in blood and tissue samples. Significant hypermethylation was observed in bladder cancer cases compared to controls ($P< 0.0001$) (Table 2).

The methylation status of CALCA and CCNA1 genes was analyzed using Methylation-Specific PCR (MS-PCR) in blood and tissue samples.66.2% ($P< 0.0001$) of bladder cancer cases exhibited CALCA gene methylation as compared to 1.25% ($P<0.0001$)of controls. 53.9% of cases had CCNA1 gene methylation, compared to 2.5% of controls ($P< 0.0001$). The association between smoking and methylation of CALCA ($P= 0.0001$) and CCNA1 ($P= 0.0038$) was significant.81.1% of smokers had CALCA methylation, compared to 44.4% of non-smokers ($P= 0.0001$).67.9% of smokers had CCNA1 methylation, compared to 33.3% of non-smokers($P= 0.0038$). Bladder cancer cases with occupational exposure to chemicals (dyes, rubber, petroleum industries) showed significantly higher methylation rates of CALCA ($P= 0.0005$) and CCNA1 ($P= 0.0015$).93.3% of cases exposed to chemicals had CALCA methylation,compared to 42.4% of non-exposed cases ($P= 0.0005$).80.0% of exposed cases had CCNA1 methylation, compared to 35.6% of non-exposed cases ($P= 0.0015$).CCNA1 gene methylation was significantly correlated with tumor recurrence
($P< 0.05$), while CALCA gene methylation showed no significant association.CCNA1 methylation was observed in 78.4% of recurrent cases, compared to 31.6% of non-recurrent cases ($P= 0.003$).CALCA

Table 1 — Primers for CALCA and CCNA1 Genes

Gene	Primer Type	Direction	Sequence
CALCA	Methylated	Forward	5′-AGTAGGCGTTTTAGTTTTAGCGC-3′
CALCA	Methylated	Reverse	5′-ACAAATACGAAAACTCCGACG-3′
CALCA	Unmethylated	Forward	5′-TTAGGTTTTAGTTTTAGTGGTGTT-3′
CALCA	Unmethylated	Reverse	5′-AACAAATACAAAAACTCCAACAC-3′
CCNA1	Methylated	Forward	5′-CGTCGCGTTTTTAGAGTTTTC-3′
CCNA1	Methylated	Reverse	5′-ACGACGAAAAACGAACTCCG-3′
CCNA1	Unmethylated	Forward	5′-TGTTTGTTTTGTAGTTTTTGTGTG-3′
CCNA1	Unmethylated	Reverse	5′-CACCTCAAAAAAACCAACTCC-3′

It shows Primers designed and used for Methylation specific Polymerase Chain Reaction(PCR)

Table 2 — Demographic and Clinical Characteristics of the Participants

Characteristic	Bladder Cancer Cases (n=89)	Controls (n=80)	p-value
Age (Mean ± SD, years)	60.56 ± 5.85	45.37 ± 10.75	<0.05
Age distribution			
≤55 years	28 (31.5%)	47 (58.75%)	<0.05
>55 years	61 (68.5%)	33 (41.25%)	<0.05
Gender			
Male	80 (89.9%)	65 (81.3%)	<0.01
Female	9 (10.1%)	15 (18.7%)	<0.01
Smoking History	53 (59.56%)	0 (0%)	-
Occupational Exposure	30 (33.7%)	0 (0%)	-
Tumor Recurrence	51 (57.3%)	-	-

It represents the demographic and clinical profile of UBC patients in comparison to healthy controls, showing statistical significance in age and gender distribution. Smoking history, occupational exposure, and tumor recurrence were reported exclusively among bladder cancer cases

Table 2 — Methylation Status of CALCA and CCNA1 Genes and their Association with Environmental and Clinical Factors

Variable	CALCA Gene Methylation (%)	p-value	CCNA1 Gene Methylation (%)	p-value
Overall Methylation				
• Cases (n=89)	59 (66.2%)	<0.0001	48 (53.9%)	<0.0001
• Controls (n=80)	1 (1.25%)		2 (2.5%)	
Smoking Status (Cases Only)				
• Smokers (n=53)	43 (81.1%)	0.0001	36 (67.9%)	0.0038
• Non-smokers (n=36)	16 (44.4%)		12 (33.3%)	
Occupational Exposure (Cases Only)				
• Exposed (n=30)	28 (93.3%)	0.0005	24 (80.0%)	0.0015
• Non-exposed (n=59)	25 (42.4%)		21 (35.6%)	
Tumor Recurrence (Cases Only)				
• Recurrence (n=51)	38 (74.5%)	0.07 (NS)	40 (78.4%)	0.003
• No Recurrence (n=38)	21 (55.2%)		12 (31.6%)	

Details the methylation status of CALCA and CCNA1 genes in UBC patients in comparison to the controls, showing associations with history of smoking, occupational chemical exposure, and tumour recurrence

methylation was higher in recurrent cases (74.5%) but was not statistically significant (NS) (P= 0.07) (Table 3).

Discussion

UBC is a significant global cancer because of its high incidence, recurrence, and progression rates. The present study evaluated the methylation status of the CALCA and CCNA1 genes in bladder cancer patients and their association with environmental factors, including smoking and chemical exposure. The results in our study clearly bring out that DNA methylation is also responsible for bladder cancer development, thus favouring the role of CALCA and CCNA1 genes as epigenetic biomarkers for early diagnosis and stratifying risk. Our study clearly indicated that CALCA and CCNA1 gene methylation was positive in bladder cancer patients than in controls (66.2% *vs*. 1.25% and 53.9% vs. 2.5%, respectively; P< 0.0001). Similar findings have been discussed in previous research, mentioning the role of silencing of tumour suppressor genes in bladder cancer progression epigenetically[2]. DNA hypermethylation of CALCA AND CCNA1 may contribute to the loss of tumour suppressor function, causing cell proliferation and tumour initiation.

Biological significance of the methylation links

DNA hypermethylation of Calcitonin/α-CGRP locus (CALCA) geneis responsible for silencing peptides that play a significant role in neuroendocrine signalling and immune modulation. In chronically exposed bladder epithelium, which is exposed to aromatic amines or other carcinogens, this silencing may turn down anti-inflammatory signalling and favour an immune microenvironment causing cell proliferation[4]. This methylation integrates with the epithelial stress caused by chronic exposure to the chemicals and renders CALCA methylation exposure responsive. CCNA1 (Cyclin A1) gene controls the

cell cycle mechanisms as well as DNA damage responses. When it gets hypermethylated, the CCNA1 expression is decreased, which disrupts the checkpoint processes and integrity, causing genomic instability[18]. This explains why CCNA1 methylation aligns with recurrence in our cohort; rather than acting as a simple driver, it may be a surrogate of a field-wide epigenetic lesion that predisposes to new/re-emergent tumours after local therapy.

Tobacco smoke and industrial aromatic amines influence one-carbon metabolism and DNA methyltransferase activity, favouring CpG island hypermethylation at promoters of tumor relevant genes. Our exposure-linked methylation (both genes) and the recurrence specific signal in CCNA1 support a model in which environmental drivers imprint durable epigenetic alterations that persist beyond resection and can herald relapse. Together, these data argue for integrating methylation testing into risk assessment, especially for smokers and chemically exposed workers and for post-TURBT surveillance, where CCNA1 methylation positivity might flag patients for intensified follow-up.

Smoking and Methylation: A Strong Correlation

Our study confirms that smoking is a significant risk factor associated with the hypermethylation of CALCA (P= 0.0001) and CCNA1 (P= 0.0038) genes. The observed correlation between smoking and epigenetic alterations is consistent with previous reports linking aromatic amines and polycyclic hydrocarbons in cigarette smoke to DNA damage and aberrant methylation[7]. The reversible nature of methylation with smoking cessation has been explored in lung cancer studies, but its impact on bladder cancer requires further investigation[18].Smoking acts as a double-edged sword, first it promotes de novo methylation, also interferes with DNA repair mechanisms, which silences the gene. This unique insight underscores the requirement for DNA methylation monitoring amongst smokers.

Occupational Exposure causing Epigenetic Alterations

The study has clearly established an association of gene methylation of CALCA and CCNA1 with industrial chemical exposure. The population working in contact with chemicals like dyes, leather, petroleum, and rubber exhibited higher methylation rates of CALCA (P= 0.0005) and CCNA1 (P= 0.0015). Prolonged duration of exposure to Industrial solvents, as well as aromatic amines, has been linked to methylation changes in urothelial cells[19]. Understanding this mechanism emphasises the need to assess workers exposed to such chemicals using methylation markers. Hence, periodic screening with such a non-invasive marker in occupational health practices could facilitate early detection of bladder cancer.

CCNA1 promoter hypermethylation as a predictor of tumour recurrence

Significant correlation between CCNA1 promoter hypermethylation and tumour recurrence was observed in our study (P= 0.003).Regulation of the cell cycle at various checkpoints and cell death mechanism is carried out by CCNA. During its hypermethylation, it may cause uncontrolled cell proliferation and rapid tumour progression[11]. These findings have been noted in earlier studies, clarifying role of CCNA1 methylation as a marker of aggressive tumour spread as well as high recurrence rate (50-70%) [21]. Hence, the need for the discovery of recurrence markers for personalised patient management. CCNA1 methylation may be deployed for risk stratification and to define prognosis in UBC patients. This can also serve as a monitoring marker, with post-treatment methylation levels offering insights into tumour relapse and helping optimise follow-up protocols.

Lack of Association with Age, Gender, and Tumour Grade

Our study analysis did not show any correlation or association between methylation status and age, gender, or tumour grade in our cohort (P> 0.05). However, some studies have reported age-related differences inDNA methylation, attributing them to cumulative exposure to environmental carcinogens. DNA methylation of the CALCA and CCNA1 genes has been closely associated with external ecological exposures rather than age-induced factors[11]. In a synergistic mechanism, the lack of association with tumour grade points out that these genes might play a role in early stages of tumour development rather than disease progression. Large scale in-depth studies should investigate the role of these molecular markers in various grades of UBC separately.

Clinical Implications

The integration of DNA molecular biomarkers is well supported by our observations in clinical diagnostics for bladder cancer, particularly for early stage diagnosis and identification of recurrence. More importantly, CCNA1 hypermethylation could be included in a risk progression strategy to guide targeted

follow-up. On top of that, the development and validation of non-invasive urine-based assays targeting CALCA and CCNA1 methylation could reduce reliance on invasive techniques such as cystoscopy for monitoring. Multicenter, large-scale, advanced trials are needed at the earliest to explore whether methylation reversal through lifestyle modifications (*e.g*., smoking cessation) or drug therapy could reduce recurrence and improve clinical outcomes, thereby improving the quality of life of UBC patients. Our study has shown a positive association between CALCA and CCNA1 gene promoter hypermethylation with environmental exposures in urothelial cancer cases. We have also observed a positive correlation between CCNA1 gene methylation with tumour recurrence, highlighting its potential as a prognostic molecular marker. These findings support surveillance in high-risk populations using epigenetic methods based on DNA methylation, which are non-invasive.

Conclusion

The study highlights the major role of epigenetic mechanisms, showing that DNA methylation of the CALCA and CCNA1 genes is strongly associated with urinary bladder cancer and is significantly influenced by lifestyle and environmental factors, such as smoking and exposure to chemicals. The promoter region hypermethylation of genes observed in UBC patients in comparison to healthy controls shows the enormous potential of these genes as epigenetic DNA-based molecular biomarkers for early diagnosis and risk identification, especially in known cases of UBC by non-invasive methods. DNA methylation of the CCNA1 gene has been observed to be strongly associated withtumour recurrence in our population, indicating its potential role as a prognostic molecular marker. The findings clearly state that epigenetic surveillance monitoring can be performed by non-invasive method in high-risk populations. These Methods could support exposure-aware risk assessment and complement established methods such as cystoscopy/urine cytology after treatment, explicitly focusing on CCNA1 positivity, which indicates a higher recurrence risk that may warrant closer follow-up.

To extend clinical utility, further studies are warranted to:

1. Develop and validate urine-based assays to detect DNA Methylation from tumour cells in a panel of genes like CALCA and CCNA1 or p16, to reduce the reliance on invasive cystoscopy.
2. Explore the reversible nature of the epigenetic mechanism of DNA methylation through lifestyle interventions, including smoking cessation or using dietary modifications.
3. Measure the sensitivity, specificity and positive predictive value of methylation markers, especially the CCNA1 gene, for tumour recurrence.
4. Understand the impact of demethylating agents in reversing the methylation.

In summary, these molecular gene-based methylation markers hold immense value as non-invasive, exposure-linked tools that can be measured at baseline and for recurrence monitoring during follow-up, pending validation in larger cohorts.

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Conflict of interest

All authors declare no conflict of interest.

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