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Mutational landscape of retinoblastoma 1(RB1) gene in bladder cancer: insights from a Bangladeshi patients cohort

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Abstract

Bladder cancer is a significant health concern in Bangladesh, yet molecular studies on its genetic drivers remain limited. The Retinoblastoma 1(RB1) gene, a pivotal tumor suppressor regulating cell cycle progression, is frequently altered in various malignancies, including bladder cancer. Mutations in exons 18, 22, and 23 of RB1 are particularly critical, as they affect key functional domains of the pRB protein. This study aimed to evaluate the mutational status of these exons in a cohort of Bangladeshi bladder cancer patients. A total of 40 histopathologically confirmed bladder cancer tissue samples were collected from patients treated at the National Institute of Cancer Research and Hospital (NICRH), Dhaka. Genomic DNA was extracted and exons 18, 22, and 23 of RB1 were amplified via PCR using specific primers. Amplicons were verified by agarose gel electrophoresis and subsequently analyzed through Sanger sequencing. High-quality sequence reads were aligned against the reference RB1 gene to identify mutations. Despite previous reports of frequent RB1 mutations in these exons in other populations, no mutations were detected in any of the 40 samples analyzed. The findings suggest possible population-specific genetic variation and indicate that other exons or regulatory mechanisms may play a more prominent role in RB1 inactivation in Bangladeshi patients. This study contributes novel data to the molecular understanding of bladder cancer in Bangladesh and highlights the need for broader genomic and epigenetic investigations to uncover relevant biomarkers in this population.

1. Introduction

Bladder cancer is one of the most common malignancies of the urinary tract, ranking as the tenth most frequently diagnosed cancer globally. In 2020, it accounted for an estimated 573,000 new cases and 213,000 deaths worldwide, with men being disproportionately affected[(1)]. It presents a significant healthcare burden, particularly in developing countries where access to diagnostic and therapeutic interventions may be limited. In Bangladesh,

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the incidence of bladder cancer is rising, yet comprehensive molecular studies focusing on the genetic landscape of the disease remain scarce.

Bladder cancer is a genetically heterogeneous disease that encompasses two main clinical subtypes: non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). The transformation from low-grade to high-grade tumors is often driven by cumulative genetic alterations affecting key regulators of cell growth and apoptosis[2]. One of the most frequently altered tumor suppressor genes in bladder cancer is RB1 (Retinoblastoma 1), a crucial gatekeeper of the G1/S transition in the cell cycle[3]. The RB1 protein (pRB) exerts its tumor suppressive function primarily by binding to E2F transcription factors and preventing uncontrolled cell proliferation. Inactivation of RB1 leads to dysregulation of cell cycle checkpoints, genomic instability, and malignant transformation. Located on chromosome 13q14, the RB1 gene spans over 180 kilobases and consists of 27 exons. Mutations, deletions, or promoter methylation of RB1 have been observed across multiple tumor types, including retinoblastoma, osteosarcoma, breast cancer, and notably, urothelial carcinoma of the bladder[4-6]. Previous studies have reported RB1 mutations in both high-grade and low-grade bladder tumors, although their frequency and impact may vary depending on the population and tumor subtype. Exons 18, 22, and 23 of the RB1 gene are particularly important, as they encode functional domains essential for the structural integrity and regulatory capacity of the pRB protein. Alterations in these exons can disrupt the interaction of pRB with E2F or other regulatory proteins, leading to a loss of tumor suppressor function[7,8]. Documented mutations at these exons from cBioPortal (https://www.cbioportal.org/) is presented in the Figure 1.

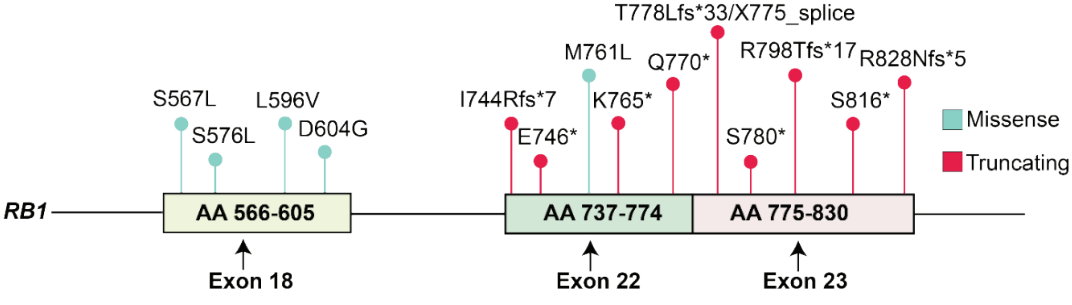

Fig. 1. Prevalent mutations in the exons 18, 22, and 23 of the RB1 gene in bladder cancer patients. *Data collected from cBioPortal (https://www.cbioportal.org/).*

While extensive research has been conducted in Western populations, there is a significant knowledge gap regarding the mutational landscape of RB1 in Bangladeshi bladder cancer patients. Local environmental exposures, genetic diversity, and healthcare disparities necessitate region-specific studies to uncover population-relevant biomarkers. A molecular understanding of bladder cancer in the Bangladeshi population could not only enhance diagnostic accuracy but also inform the development of targeted interventions and personalized therapies.

Given this context, the present study aimed to investigate the presence of mutations in exons 18, 22, and 23 of the RB1 gene in bladder cancer patients from Bangladesh. Using PCR amplification and Sanger sequencing, we analyzed tumor tissue DNA samples to determine whether these exonic regions harbor clinically relevant alterations. The findings of this study are expected to contribute to the growing body of molecular data on bladder cancer and support future translational research in cancer genomics within the region.

2. Materials and Methods

2.1. Study design

This study aimed to identify mutations in exons 18, 22, and 23 of the RB transcriptional corepressor 1 (*RB1*) gene in Bangladeshi bladder cancer patients. DNA was extracted from bladder tissue samples collected from 40 patients. The target exons were amplified by PCR using two sets of specific primers. The sizes of the PCR amplicons were confirmed by agarose gel electrophoresis. Subsequently, the amplicons were subjected to Sanger sequencing, and the resulting sequences were analyzed to identify any prevalent mutations associated with bladder cancer.

2.2. Study population

This study was conducted on 40 Bangladeshi bladder cancer patients. Informed consent was obtained from all participants, and written consent forms were collected. Tissue samples were obtained from patients admitted to the Urology Department of the National Institute of Cancer Research and Hospital (NICRH), Mohakhali, Dhaka, Bangladesh, between January 2021 and February 2024.

2.3. Sample collection and storage

Tissue biopsies were obtained during routine surgical procedures (e.g., transurethral resection or cystectomy) and were immediately placed in sterile, RNase/DNase-free cryovials. Each sample was submerged in 1 mL of RNAlater stabilization solution to preserve nucleic acid integrity and stored at 4°C overnight. The next day, samples were transferred to −80°C for long-term storage until further molecular processing.

2.4. Histopathological confirmation

All bladder tissue specimens collected for this study underwent histopathological examination to confirm the diagnosis and classify tumor grade. After surgical excision, tissue samples were fixed in 10% neutral-buffered formalin, processed using standard paraffin embedding protocols, and sectioned at 4 μm thickness. The sections were stained with hematoxylin and eosin (H&E) and evaluated by experienced pathologists at the National Institute of Cancer Research and Hospital (NICRH), Dhaka, Bangladesh.

Tumor grading was performed according to the 2016 World Health Organization/ International Society of Urological Pathology (WHO/ISUP) classification system for urothelial neoplasms. Based on morphological features such as architectural disorganization,

nuclear atypia, and mitotic activity, tumors were categorized as either low-grade or high-grade urothelial carcinoma. Only samples with confirmed diagnosis of bladder cancer and clearly defined histological grading were included in the study.

2.5. DNA extraction

Genomic DNA was extracted from bladder tissue samples using the QIAgen Puregene Core Kit A (Qiagen, Germany), following the manufacturer's protocol with slight modifications. Briefly, approximately 25 mg of tissue was homogenized and lysed in Cell Lysis Solution containing proteinase K, and incubated at 55°C until complete digestion was achieved. RNA was removed by treatment with RNase A. Protein precipitation was carried out using the provided Protein Precipitation Solution, followed by centrifugation to separate the protein pellet. The supernatant containing DNA was transferred to a fresh tube, and DNA was precipitated using isopropanol. The DNA pellet was then washed with 70% ethanol, air-dried, and resuspended in DNA Hydration Solution. The quality and concentration of the extracted DNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

2.6. PCR amplification of RB1 exons

Exons 18, 22, and 23 of the *RB1* gene were amplified using conventional polymerase chain reaction (PCR). Two sets of primers were designed to specifically target these exons. Primer set 1 was used to amplify exon 18, while Primer set 2 was used to simultaneously amplify exons 22 and 23. Primer set 1: F: CTGTCTTTCCTCCTAACTTCTAAC, R: CTGCCATGAAAACTGCTCTCA. Primer set 2: F: GAGCAGCTATAATCCAAGCCT, R: TAGATTTTCTTCACCCCGCC. PCR reactions were performed in a 25 μL volume containing 12.5 μL of 2X PCR Master Mix (Thermo Scientific, USA), 0.5 μL of each primer (10 μM), 2 μL of genomic DNA (approximately 50–100 ng), and nuclease-free water to adjust the final volume. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at optimized temperatures (57°C for Primer set 1 and 57.5 °C for Primer set 2) for 30 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 7 minutes.
The PCR products were then confirmed by electrophoresis on a 2% agarose gel stained with Midori Green and visualized under UV transillumination.

2.7. Sanger sequencing and data analysis

The PCR amplicons were sequenced using the Sanger sequencing method. Raw sequence reads were quality-trimmed based on Phred scores, retaining only those regions with a Phred score of 40 or higher, corresponding to a base call accuracy of 99.99% (0.01% error rate). The high-quality trimmed sequences were then aligned to the reference RB1 gene sequence using Geneious Prime software. The aligned sequences were carefully analyzed to identify any potential mutations.

3. Results and Discussion

3.1. Demographic characteristics of study participants

This study included 40 bladder cancer patients from Bangladesh. Among them, 21 cases (52.5%) were diagnosed with high-grade bladder cancer, while the remaining 19 cases (47.5%) were classified as low-grade. The participants had an average age of 57.6 years (±10.83), with a median age of 60 years. The mean body mass index (BMI) was 21.96 ± 3.40 kg/m², and the median BMI was 21.62 kg/m². Age distribution revealed that the highest proportion of patients (35%) fell within the 60–69 age group, followed by 20% in the 50–59 range, 27.5% in 70–79, 12.5% in 40–49, and 5% in the 30–39 age group. Of the total patients, 34 (85%) were male and 6 (15%) were female. Demographic characteristics of the study participants is represented in the Table 1, and Figure 2.

Table 1. Demographic characteristics of bladder cancer patients in this study

Characteristics		Frequency (n)	Percentage (%)
Age (years)	Mean (±SD)	57.6 (±10.83)	
	Median	60	
Age group (years)	30-39	2	5
	40-49	5	12.5
	50-59	8	20
	60-69	14	35
	70-79	11	27.5
BMI	Mean (±SD)	21.96 (±3.40)	
	Median	21.62	
Gender	Male	34	85
	Female	6	15
Tumor Grade	High Grade	21	52.5
	Low Grade	19	47.5
Other Cancer	Yes	6	15
	No	33	82.5
	N/A	1	2.5
Diabetes mellitus type 2	Yes	7	17.5
	No	31	77.5
	N/A	2	5
Hematuria	Yes	36	90
	No	4	10
Kidney stone	Yes	5	12.5
	No	31	77.5
	N/A	4	10
Smoking	Yes	29	72.5
	No	11	27.5

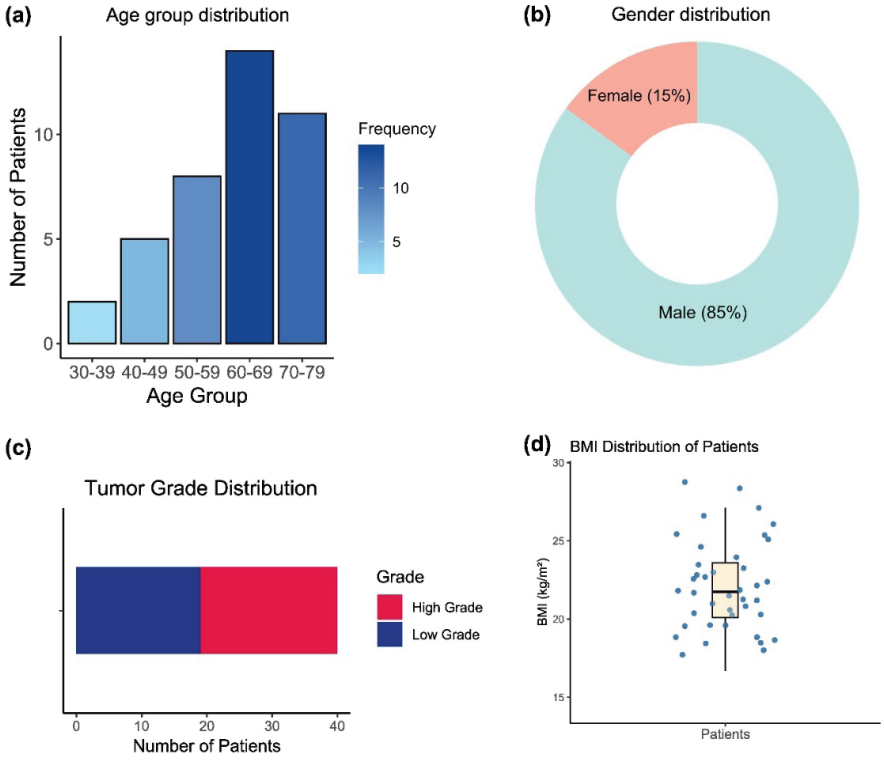

Fig. 2. Demographic characteristics of the bladder cancer patients enrolled in this study.

(a) Age group distribution: The majority of patients (36.67%) were within the 60–69 years age range, followed by 50–59, 70–79, 40–49, and 30–39 years. **(b)** Gender distribution: A donut chart illustrating that 85% of the patients were male and 15% were female. **(c)** Tumor grade distribution: A horizontal stacked bar chart showing the distribution between high-grade (52.5%) and low-grade (47.5%) bladder cancer cases. **(d)** BMI distribution: A box plot representing the range and median of BMI values among the 40 patients, with the average BMI being approximately 21.96 ± 3.40 kg/m².

3.2. Histopathological confirmation of tumors grade

The tumor grade was determined from the histopathology report done for 40 samples. Hematoxylin and eosin (H&E) staining is used in this study to investigate the histopathological distinctions between high-grade (HG) and low-grade (LG) tumors. Tissue samples analyzed under a microscope showed unique morphological traits that distinguished tumor grades (Fig. 3). Lower cellular density and well-organized structures with preserved differentiation were observed in LG tumors. The aggressive behavior of HG tumors, on the other hand, was indicated by their high mitotic activity, loss of structural integrity, and notable pleomorphism.

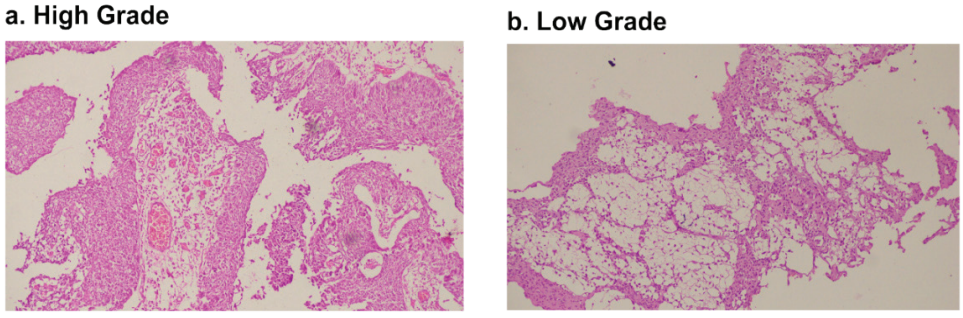

Fig. 3. Histopathology of tumor samples.

Representative histopathological images of high-grade (HG) and low-grade (LG) bladder cancer tissues. HG samples show marked cellular atypia, high mitotic activity, and disorganized architecture, while LG samples exhibit relatively uniform cells with minimal atypia and preserved urothelial layering. These morphological differences support the classification and grading used in the study.

3.3. PCR amplicons

Exon 18 of the RB1 gene was amplified using Primer Set 1, while exons 22 and 23 were amplified using Primer Set 2. The expected amplicon sizes were 619 bp for Primer Set 1 and 735 bp for Primer Set 2. Successful amplification of the target regions was confirmed by electrophoresis on a 2% agarose gel. The sizes of the PCR amplicons are illustrated in Figure 4.

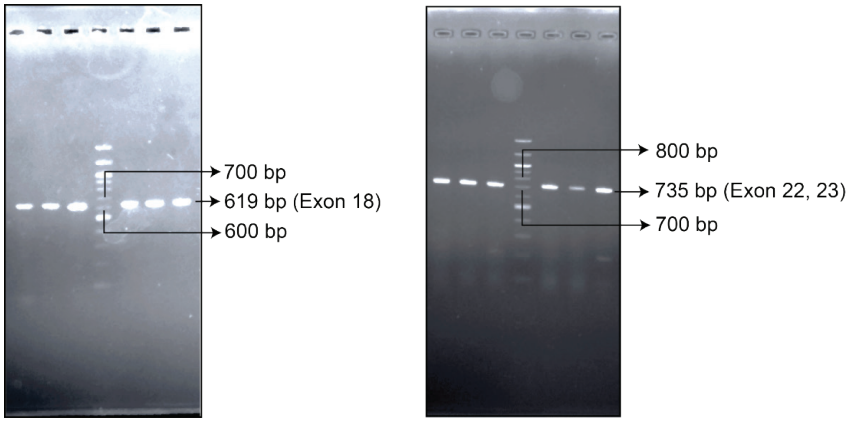

Fig. 4. Agarose gel electrophoresis of PCR amplicons.

PCR products were separated on a 2% agarose gel and stained with Midori Green for visualization. Comparison with a 100 bp DNA ladder confirmed successful amplification of the target regions: a 619 bp fragment corresponding to exon 18 (amplified with Primer Set 1) and a 735 bp fragment representing exons 22 and 23 (amplified with Primer Set 2) of the *RB1* gene.

3.4. Sanger sequencing and analysis

Sequences obtained from Sanger sequencing were quality-trimmed based on Phred scores, retaining only those with a confidence level corresponding to 99.99% base call accuracy. These high-quality sequences were then used for downstream analysis. Figure 5 shows representative chromatograms for exon 18 and exons 22 and 23 of the *RB1* gene.

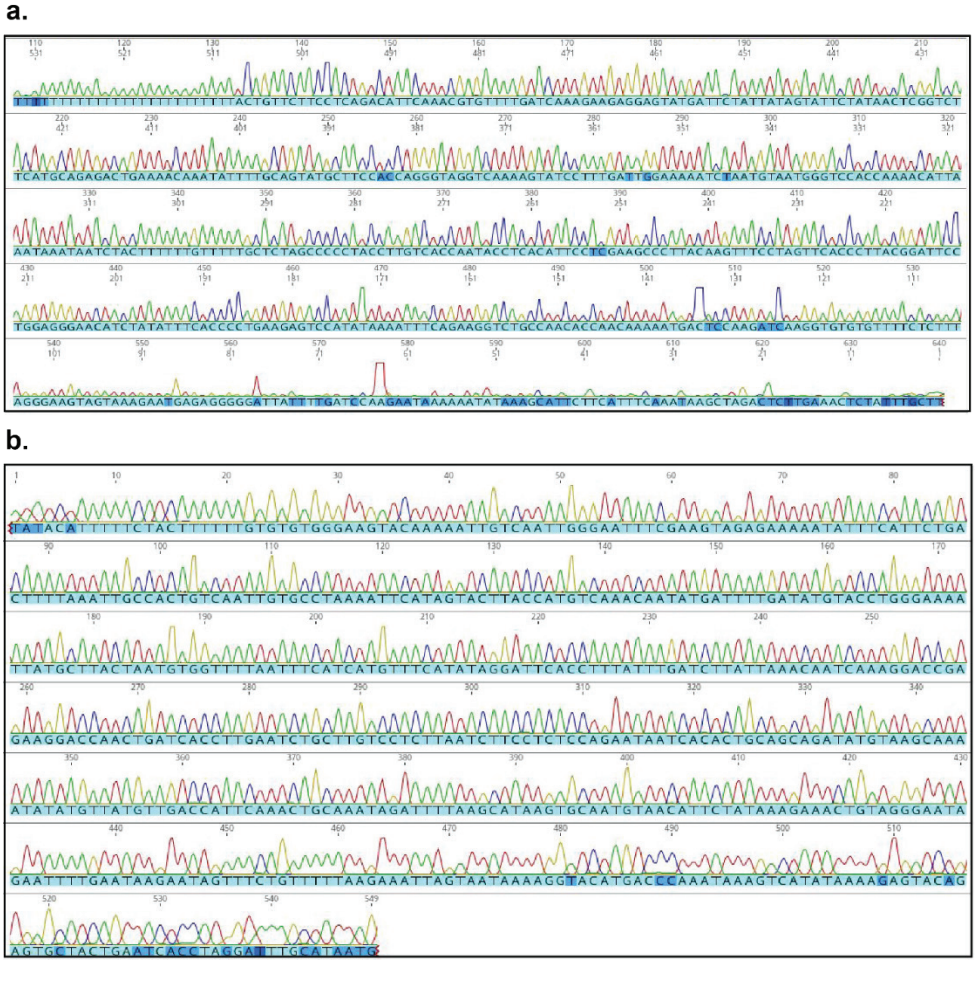

Fig. 5. Representative chromatograms of PCR amplicon sequences.

(a) Chromatogram of the RB1 gene exon 18 sequence. (b) Chromatogram of the RB1 gene exons 22 and 23 sequence.

Alignment of the processed sequences with the *RB1* reference sequence revealed no mutations in exons 18, 22, or 23 among the analyzed samples.

In this study, we investigated the mutational status of exons 18, 22, and 23 of the RB1 gene in 40 Bangladeshi bladder cancer patients using PCR amplification followed by Sanger sequencing. Despite extensive documentation of RB1 mutations in bladder cancer across global cohorts, our findings revealed no detectable mutations in the examined exons among the studied patients. This result contributes novel region-specific insights to the mutational landscape of bladder cancer and suggests that exons 18, 22, and 23 of RB1 may not represent mutation hotspots in this population.

The RB1 gene encodes a critical tumor suppressor protein (pRB) that regulates the G1 to S phase progression of the cell cycle by binding to E2F transcription factors and inhibiting uncontrolled proliferation[9,10]. Loss of RB1 function has been implicated in a wide range of malignancies including retinoblastoma, osteosarcoma, lung cancer, and urothelial carcinoma of the bladder[11-13]. In bladder cancer, RB1 inactivation is particularly associated with progression from non-muscle-invasive (NMIBC) to muscle-invasive disease (MIBC) and poorer clinical outcomes[14,15].

Previous genomic studies have reported *RB1* alterations such as point mutations, deletions, and promoter hypermethylation in a significant proportion of high-grade bladder cancers, with mutation frequencies ranging from 10–25% depending on the cohort and methodology[16,17]. Notably, mutations affecting exons 18, 22, and 23 have been functionally linked to disruption of RB1's C-terminal domain, which is essential for its tumor suppressor activity[18]. Data from large-scale cancer databases such as cBioPortal also indicate recurrent mutations in these exons in bladder tumors from Western populations.

However, in contrast to these findings, our study did not identify any mutations in these exons among the 40 Bangladeshi bladder cancer patients. Several explanations may account for this discrepancy. First, population-specific genetic variation may influence the mutational spectra of RB1, and prior genomic studies have predominantly focused on Western or East Asian cohorts, with limited data from South Asia. Second, the relatively small sample size of our study may limit the detection of low-frequency variants. Third, RB1 alterations may occur in other exonic regions or through structural variations (e.g., large deletions or promoter methylation) that were not assessed in this study.

Our demographic data showed that the majority of patients were male (85%) and aged between 50–79 years, which aligns with established epidemiological patterns of bladder cancer[19]. High-grade tumors comprised 52.5% of the cohort, indicating a predominance of clinically aggressive disease. This underscores the importance of identifying molecular biomarkers to support early diagnosis and risk stratification. While we did not observe mutations in the selected RB1 exons, this negative result is still valuable, as it suggests the need to explore other exons, or complementary tumor suppressor genes such as *TP53*, *CDKN2A*, or *PTEN* in Bangladeshi patients.

Our findings also raise questions about the role of non-mutational mechanisms in RB1 downregulation, such as promoter hypermethylation or post-transcriptional silencing via microRNAs, which have been shown to contribute to pRB loss in various cancers[20,21]. These avenues warrant further investigation in the Bangladeshi context.

In conclusion, although no mutations were detected in exons 18, 22, or 23 of the RB1 gene in this cohort, this study provides a foundation for future molecular investigations in bladder cancer among Bangladeshi populations. It emphasizes the necessity for larger-scale, multi-omic studies to uncover actionable biomarkers that may guide personalized therapy and improve patient outcomes in Bangladesh.

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