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Research article

Delineating the mechanistic relevance of the *TP53* gene and its mutational impact on gene expression and patients' survival in bladder cancer

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ABSTRACT

Bladder carcinoma (BLCA) is a widespread urological malignancy causing significant global mortality, often hindered by delayed diagnosis and limited treatments. BLCA frequently exhibits *TP53* mutations, playing a pivotal role in its pathogenesis and underscoring the potential of targeting *TP53* as a therapeutic approach for this prevalent urological malignancy. Tumor tissues from 50 bladder cancer patients were used for mutational analysis in *TP53*'s mutation-rich exons (5, 7, & 8). The gene expression of the *TP53* gene, along with a *TP53*-target gene B-cell translocation gene 2 (*BTG2*) was also assessed in the cDNA samples from the same BLCA tissues and 15 urine controls of healthy people. The analysis revealed 22 % of patients with somatic hotspot mutations, 18 % with pathogenic missense mutations, and 12 % with intronic variants. Patients with somatic mutations exhibited the worst prognosis, supported by survival analysis from The Cancer Genome Atlas (TCGA) BLCA data. Interestingly, H296Y missense mutation correlated with higher *TP53* expression and improved survival, while intronic SNPs were linked to worse outcomes. Additionally, upregulated *BTG2* expression in mutated patients was observed which was correlated with poor prognosis, emphasizing the role of *TP53* mutations in bladder cancer progression. The multivariate analysis highlighted the predictive power of *TP53* mutations, with a high frequency of high-grade tumors (78.57 %) in mutated patients, underscoring their role in cancer progression. In conclusion, this study emphasizes the crucial role of *TP53* mutations in bladder cancer patients from Bangladesh.

1. Introduction

The fatality rate from bladder carcinoma, which is the tenth most common cancer worldwide, is 0.84 % per 100,000 persons in Bangladesh [1]. Each year, around 573000 new cases are diagnosed globally, leading to 213000 fatalities [2]. When aberrant cells in the bladder begin to proliferate uncontrolled and form a tumor, bladder cancer results. These tumors can encroach on the bladder wall and then spread via the bloodstream or lymphatic system to other regions of the body. The most prevalent form of bladder cancer is

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transitional cell carcinoma (TCC), sometimes referred to as urothelial carcinoma. Smoking, exposure to certain chemicals, such as those used in the dye, rubber, and leather industries, and a family history of the illness are risk factors for bladder cancer. Bladder cancer is more likely to affect males than women, and the risk rises with age.

Early detection of bladder cancer is crucial for successful treatment, and various tests and techniques are employed for this purpose. Cystoscopy involves using a specialized tube to examine the urethra and bladder. Biopsy or Transurethral Resection of Bladder Tumor (TURBT) can be performed during cystoscopy to extract a cell sample for diagnosis and treatment. Urine cytology involves examining cells in a urine sample to detect cancer cells. Imaging tests like retrograde pyelogram and CT urogram provide detailed views of the urinary tract to identify potential cancerous regions. Early detection through these methods significantly improves the chances of successful treatment outcomes for bladder cancer [3].

In the pursuit of understanding this hallmark of cancer, extensive research has unveiled a trio of pivotal suppressive genes that play a pivotal role in inhibiting cellular growth and proliferation. These genes, namely Tumor protein p53 (*TP53*), Phosphatase and tensin homolog (PTEN), and retinoblastoma (*RB*), serve as guardians of orderly cell division, ensuring that it occurs in a highly regulated manner in non-cancerous cells [4,5]. *TP53* has emerged as a pivotal tumor suppressor across various cancer types, managing a multifaceted role in navigating complex cellular progression. *TP53* mutations are prevalent in bladder cancer, with nearly half of the muscle-invasive cases showing these mutations, compromising *TP53* function in 76 % of cases. These mutations, along with *TP53*-associated pathways, drive bladder cancer progression, impacting prognosis and guiding therapeutic approaches [6–8]. About 7 % of bladder cancer cases have been linked to heritable genetic predispositions [9].

TP53 controls the regulated expression of many genes, including *BTG2* which is another tumor suppressor gene, and any disruption in their regulation leads to cancer development. The *BTG2* gene plays a crucial role in cancer by acting as a tumor suppressor. It regulates the cell cycle, induces programmed cell death (apoptosis), repairs DNA damage, and promotes cellular senescence, all of which help prevent uncontrolled cell proliferation and tumor growth. In a recent study, a compelling revelation emerged regarding *BTG2*, which was initially recognized as a gene under the influence of the tumor suppressor protein p53 [10]. This intricate interplay between p53 and *BTG2* sheds light on the molecular response to DNA damage and the regulatory mechanisms that govern gene expression. It highlights the significance of p53 not only as a guardian of genomic integrity but also as a key modulator of genes like *BTG2*, which can play critical roles in cellular processes and responses to stress. However, in a study, it was found that *BTG2*, typically a tumor suppressor gene, paradoxically promotes bladder cancer cell migration [11].

Mutant *TP53* further fuels mechanisms underlying cancer initiation and progression, contributing to unfavorable disease outcomes [12,13]. Indeed, research has underscored the capacity of mutant *TP53* to accelerate the proliferation of metastatic tumor cells and enhance their metastatic potential [14]. Moreover, *TP53* status has implications for chemotherapy responses and drug sensitivity in bladder cancer [15,16].

This novel study aimed to explore the complex correlation between *TP53* gene mutations and the progression of bladder cancer by examining their influence on *TP53* and the *TP53*-target gene *BTG2* expression. The study also sought to investigate the association of these mutations with the survival of bladder cancer patients within a cohort residing in Bangladesh.

2. Method and materials

This extensive study was designed with the primary aim of uncovering somatic mutations within the three most mutation-prone exon regions of *TP53* while simultaneously understanding the intricate complexities of *TP53* gene expression in individuals identified with bladder cancer. Beyond this, this research endeavor was dedicated to unraveling the modified mechanisms governing *TP53* in the context of bladder cancer, catalyzed by the acquisition of somatic *TP53* mutations. To observe this complicacy, the expression

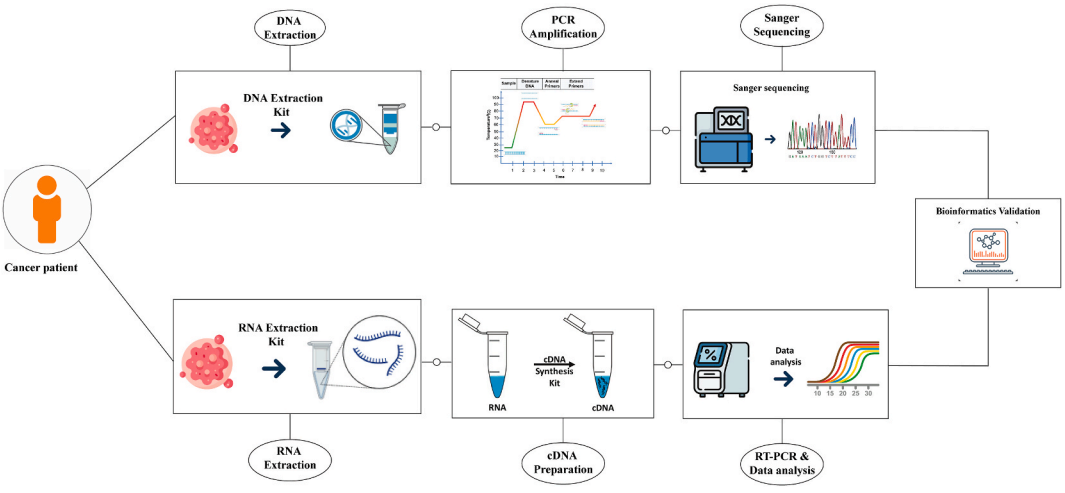

Fig. 1. Graphical representation of laboratory experiments and bioinformatics validation.

patterns of *TP53*'s target genes *BTG2* were scrutinized, thereby providing a comprehensive outcome of the repercussions stemming from *TP53* mutations and the subsequent alterations in gene expression within the realm of bladder cancer patients.

The workflow of this research is visualized in Fig. 1.

2.1. Study Subjects and sample processing

The study enrolled a cohort of 50 bladder cancer patients, regardless of gender or grade, who provided their written consent. These patients were under the care of the Urology department at the National Institute of Cancer Research and Hospital in Mohakhali. Urine samples were procured the day before the surgical procedures. Immediately following the surgical procedures, tumor samples were carefully collected within specialized RNA Protect tissue tubes at the Urology department of the National Institute of Cancer Research and Hospital in Mohakhali (Ethical approval reference: NICRH/Ethics/2021/181). These samples were then transported to the laboratory within an ice box, preserving a constant temperature range of 4-8 °C. Upon arrival at the laboratory, the tumor samples, securely nestled within RNA Protect tubes, were stored in the container of a −80 °C refrigerator, ensuring their optimum condition and readiness for future utilization in the research endeavors.

This study also included urine samples from 15 healthy individuals as control to compare expression data with tumor. The urine sample was initially collected in a 50 ml Falcon tube and transported to the laboratory at room temperature. Subsequently, the samples underwent centrifugation at 3000 rcf for 30 min at 25 °C using the Sigma 3-18k centrifuge. The resulting supernatant was discarded. The urine pellet received urine conditioning buffer from the Zymo Research Quick-DNA Tm Mini Kit, and phosphate buffer saline to retain the integrity of nucleic acid. The tube containing the pellet was vortexed until complete dissolution, followed by immediate storage at −20 °C for subsequent experiments.

2.2. Genomic DNA extraction

The extraction process was carried out utilizing the Qiagen Puregene Core Kit A, which came equipped with pre-prepared and ready-to-use reagents for convenience. Tumor tissue, initially stored at −80 °C, was thawed on ice, and 30 mg was transferred to a mortar for pulverization with liquid nitrogen. The resulting tissue paste was mixed with 900 μL of cell lysis solution, transferred to a microcentrifuge tube, and incubated at 55 °C overnight. Subsequent steps involved the addition of tissue Proteinase K, RNase, and Tissue protein precipitation solution, followed by centrifugation. The supernatant was mixed with isopropanol, centrifuged, and the DNA pellet air-dried before being rehydrated with DNA hydration solution. After incubation at 65 °C, DNA concentration and purity were assessed using Nanodrop OneC Micro volume UV–Vis Spectrophotometer (Thermo Fisher Scientific, US), and the DNA was stored at −80 °C for future use. The overall DNA extraction procedure from tumor tissue is graphically shown in Supplementary Fig. 1A.

2.3. Assessment of mutational status in the TP53 gene

In order to detect mutations within the TP53 exon regions, amplifying these specific regions through polymerase chain reaction (PCR) was performed. Using primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) from NCBI, primer pairs were created for the *TP53* gene's GenBank reference sequence (NG_017013.2) that enabled the amplification of the target sequences around exons 5, 7, and 8 in 50 patients. The primer sequences are stored in Supplementary Table 1. UCSC in Silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) has been utilized to validate the selected primer's accuracy. Amplification was performed on the Biometra TAdvance PCR system. The conditions for PCR amplification for required regions are mentioned in Supplementary Table 1. To confirm the specificity and accuracy of the PCR amplification, agarose gel electrophoresis was employed. Following the electrophoresis run, the gel was visualized using a gel documentation machine. The observed DNA bands were then compared to the DNA ladder to confirm the size of the amplicons. PCR products were purified and the Sanger sequencing was performed using state-of-the-art technology, specifically capillary gel electrophoresis, facilitated by the AB135000 genetic analyzer. Upon the successful sequencing of all the PCR product samples, the ensuing data analysis was conducted with the sophisticated Geneious Prime software V2023, available at https://www.geneious.com/prime/. In this comprehensive analysis, the sequences were meticulously aligned with a reference sequence sourced from the esteemed NCBI nucleotide reference sequence database.

2.4. RNA extraction from tumor tissue and urine pellet

RNA was extracted from 50 bladder patient tumor tissues using the Pure Link RNA extraction mini kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The RNA extraction process from tumor tissue involved thawing the tissue on ice, transferring approximately 30 mg to a mortar, and grinding it into a paste with liquid nitrogen. Subsequently, a lysis buffer with 2-mercaptoethanol was added, and after an overnight incubation at 4 °C, the sample underwent centrifugation. The RNA-containing supernatant was collected, and ethanol was added to the tissue homogenate. The mixture was processed through a spin cartridge, with wash buffers I and II applied in subsequent steps. After centrifugation to dry the membrane, RNase-free water was added for RNA elution. The eluted RNA was then stored at −80 °C for future use. The detailed process involved various centrifugation and wash steps to ensure the extraction of high-quality RNA from the tumor tissue. The RNA extraction procedure from tumor tissue was presented graphically in Supplementary Fig. 1B.

For extraction of RNA from healthy control urine centrifugation was performed following the collection of urine and supernatant was discarded to collect the urine pellet. The RNA extraction process began with the addition of 1 ml triazole reagent to a 250 ml pellet

sample, followed by pipetting for homogenization and a 5-min incubation. Chloroform (0.2 ml) was then added, and after thorough mixing, the solution was incubated for an additional 2–3 min. Centrifugation at 12000*g* for 15 min at 4 °C separated the aqueous phase, which was transferred to a new tube. Isopropanol (0.5 ml) was added, and the solution was incubated for 10 min at 4 °C before centrifugation. After discarding the supernatant, the RNA pellet underwent resuspension in 1 ml of 75 % ethanol, followed by vortexing and centrifugation. This ethanol wash step was repeated once. The RNA pellet was air-dried for 5 min, resuspended in 20–50 μL RNase-free water with 0.1 Mm EDTA, and incubated at 55–60 °C for 10–15 min. Finally, the RNA was stored at −80 °C.

The quality and quantity of the extracted RNA were evaluated using the NanoDrop OneC Micro volume UV–Vis Spectrophotometer (Thermo Fisher Scientific, US). The RNA extraction procedure from the urine pellet was presented graphically in Supplementary Fig. 1C.

2.5. Real-time quantitative PCR (RT-qPCR) and data analysis

To generate cDNA from tumor and urine-derived RNA, the ProtoScript® II First Strand cDNA Synthesis Kit was used, sourced from New England Biolabs in Ipswich, Massachusetts, United States. the TB Green qPCR Master Mix, forward and reverse primers, and template cDNA were combined to create a 10 μL reaction mixture and RT-qPCR was done using The CFX96 Real-Time system. The qPCR amplification conditions are provided in Supplementary Table 1.

In qPCR, genes of interest were amplified, TP53, and BTG2, along with a reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The RT-qPCR primers are given in Supplementary Table 1. The qPCR analysis was conducted with triplicates for each sample. Each qPCR plate included both target and housekeeping genes and the resulting data were subjected to analysis. To analyze gene expression, the ΔCT method was employed [17]. This involved obtaining CT values for *TP53, BTG2*, and *GAPDH*, and then calculating ΔCT by subtracting the CT value of GAPDH from the CT value of *TP53* and *BTG2*. Mathematically, ΔCT can be represented as follows:

ΔCT = CT of *TP53* or *BTG2* - CT of *GAPDH* (Mean values were used for CT calculations).

2.6. Statistical data analysis

Statistical analysis was thoroughly carried out using the versatile R programming language. Within the R environment, Kaplan-Meier curves, executing the Log-rank test, and conducting Wilcoxon rank sum tests were performed. In this analysis, statistical significance was attributed to P-values less than 0.05, signifying the rigor of the findings. The parameter under scrutiny was overall survival (OS), defined as the duration from diagnosis to the ultimate follow-up or demise. To assess disparities in survival curves, the log-rank test was adeptly applied. Furthermore, R programming facilitated the creation of compelling graphical representations of the data, ensuring clarity and effective communication of research findings.

Table 1
Demographic characteristics of Bladder cancer patients and healthy controls.

Demographic characteristics of Bladder cancer patients.				
Bladder Cancer Patients	Age (Years)		BMI	
	Mean (±SD)	Median (IQR)	Mean (±SD)	Median (IQR)
Male	57.51 (±11.72)	60 (17)	21.83 (±2.80)	21.71 (±2.38)
Female	57.27 (±12.09)	57 (18)	21.94 (±2.55)	21.71 (±2.22)
Characteristics		**Condition**		**No. of patients**
Cigeratte or betel leaf habit		Yes		38
		No		12
Cancer status		Recurrent		31
		Primary		19
Cancer Subtype		NMIBC		48
		MIBC		2
Cancer grade		High grade		17
		Low grade		6
		N/A		24
Hematuria		Yes		45
		No		5
Demographic characteristics of Healthy individuals.				
Healthy Controls	Age (Years)		BMI	
	Mean (±SD)	Median (IQR)	Mean (±SD)	Median (IQR)
Male	52.79(±10.57)	52(13)	24.99 (±3.73)	23.83 (±5.02)
Female	52.6(±10.57)	52 (5)	26.64 (±3.05)	26.64 (±2.15)

2.7. Computational analysis for validation

TCGA bladder carcinoma (BLCA) data was used for mutation, expression, and survival analyses to validate the outcome of experimental analyses. Swiss model was used to predict the 3D model of *TP53* mutated proteins using PDB ID: 8F2H and the Galaxy refine tool was employed for structure refinement [18,19]. PROCHECK validation tools to observe the quality of predicted models [20]. Subsequently, PyMol software and TM-align server were exploited to observe structural deviation of *TP53*-mutated models from wild *TP53* structure [21,22]. Furthermore, various prediction tools, including SIFT, PON-P2, I-Mutant, and MUpro were utilized for functional and stability observations of mutated *TP53* [23–26]. Clinvar and Cosmic databases were exploited to examine the clinical significance of these somatic mutations [27,28].

3. Result

In this study, transurethral resection of bladder tumor (TURB-T) surgery was performed on 50 bladder cancer patients. Each patient's clinical data as well as tumor tissue samples were obtained throughout the operation. The *TP53* mutation-prone exons (5,7, & 8) underwent a mutational study using Sanger sequencing on the tumor DNA. The survival rates of patients and general pathophysiological conditions were then connected with the existence of these genetic anomalies. The expression levels of the *TP53* and *TP53*-target gene *BTG2* were measured using real-time quantitative PCR in the patients to investigate the mutational impact in bladder cancer patients. The experiment also included the correlation with immune cell infiltration.

3.1. Clinicopathological features of bladder cancer patients and healthy controls

This study comprised 50 bladder cancer patients, consisting of 37 males (74 %) and 13 females (26 %). The demographic characteristics of the study participants are summarized in Table 1. For male patients, the average age was 57.51 years [±11.72 standard deviation (SD)], with a median of 60 years (interquartile range: 17). In contrast, female patients had an average age of 57.27 years (±12.09 SD) and a median of 57 years (IQR: 18). In terms of body mass index (BMI), male patients had an average BMI of 21.83 kg/m2 (±2.80 SD) and a median BMI of 21.71 kg/m2 (IQR: 2.38). On the other hand, female patients exhibited an average BMI of 21.94 kg/m2 (±2.55 SD) and a median BMI of 21.71 kg/m2 (IQR: ±2.22).

This study also included 15 healthy controls with 12 male and 3 female. The demographic characteristics of the study participants are summarized in Table 1. For male controls, the average age was 52.79 years, with a median of 52 years. In contrast, female controls had an average age of 52.6 years and a median of 52 years. In terms of body mass index (BMI), male controls had an average BMI of 24.99 kg/m2 and a median BMI of 23.83 kg/m2. On the other hand, female controls exhibited an average BMI of 26.64 kg/m2 and a

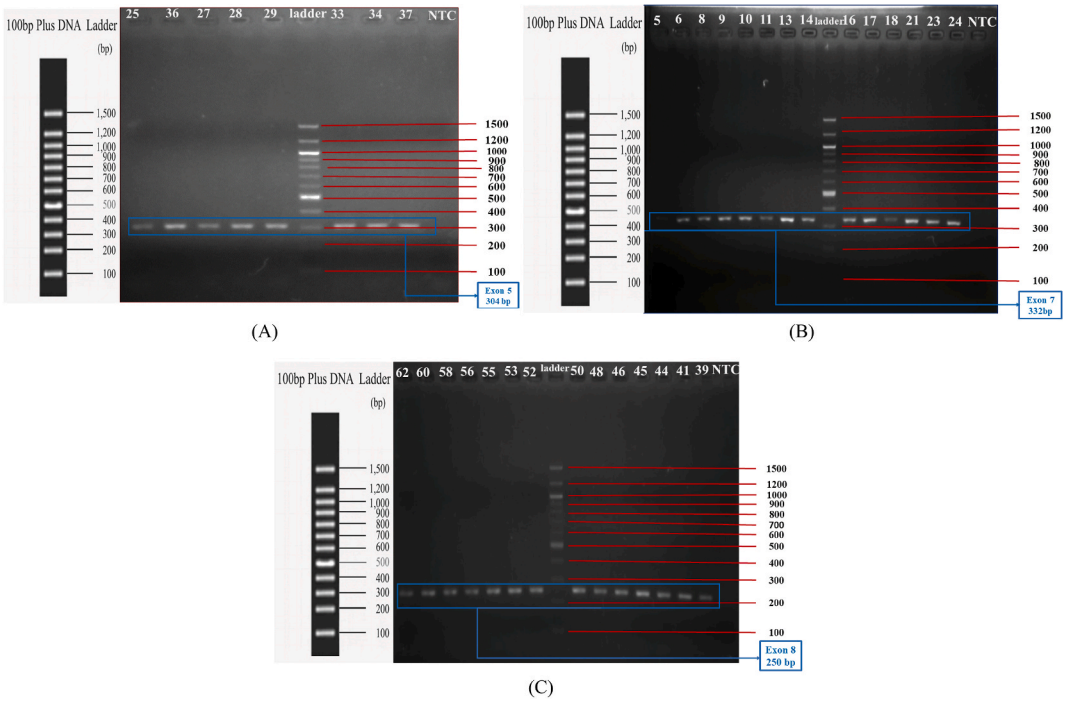

Fig. 2. Electrophoresis of PCR amplification product of *TP53* (A) exon 5 (304bp), (B) exon 7 (332 bp), and (C) exon 8 (250 bp) on agarose gels. A 100bp DNA ladder was utilized to determine the size of the DNA bands. Numbers correspond to the sample numbers, while NTC denotes to Non-Template Sequence.

median BMI of 26.64 kg/m2.

3.2. PCR amplification of exon 5,7, & 8 of TP53 gene

After optimizing the accurate condition using gradient PCR, most mutation-prone exons 5, 7, and 8 of *TP53* were amplified by polymerase chain reaction (PCR), and the reaction produced 304, 332, and 250bp products for exons 5, 7, and 8, respectively (Fig. 2). The PCR products underwent purification using the ExoSAP reagent with 100 % efficiency, after which the purified samples were subjected to sequencing using Sanger's dideoxy chain termination method.

3.3. Mutational data analysis

The Sanger sequencing data was processed using the Geneious Prime software, aligning it with the reference sequence of the *TP53* exon 5, 7, and 8 regions sourced from NCBI. Chromatogram observation of the mutations was visualized in Supplementary Fig. 2.

Mutation analysis of exons 5, 7, and 8 of the *TP53* gene revealed the presence of a total of 10 mutations including, somatic hotspot mutations, missense pathogenic mutations, and intronic variants. These 10 mutations were presented in 23 patients from a total of 50 patients giving a mutation frequency of 46 % (Fig. 3A). Mutations were presented in males in a higher proportion compared to females (Fig. 3B). Among, 10 mutations, 7 somatic mutations were discovered in 3 exons (2 in exon 5, 2 in exon 7, and 3 in exon 8) which presented in 11 bladder cancer patients giving a Somatic hotspot mutation frequency of 22 %. Moreover, one missense pathogenic mutation in exon 8 was found in 9 patients with a frequency of 18 %. Two intronic variants were found downstream of exon 7 according to hg38 in 6 patients (12 %) (Fig. 3C). Considering only pathogenic mutations, including somatic and missense mutations gave a mutation frequency of about 40 %. The details about these mutations are given below in Table 2. A graphical representation of mutational data observation is depicted in Fig. 4.

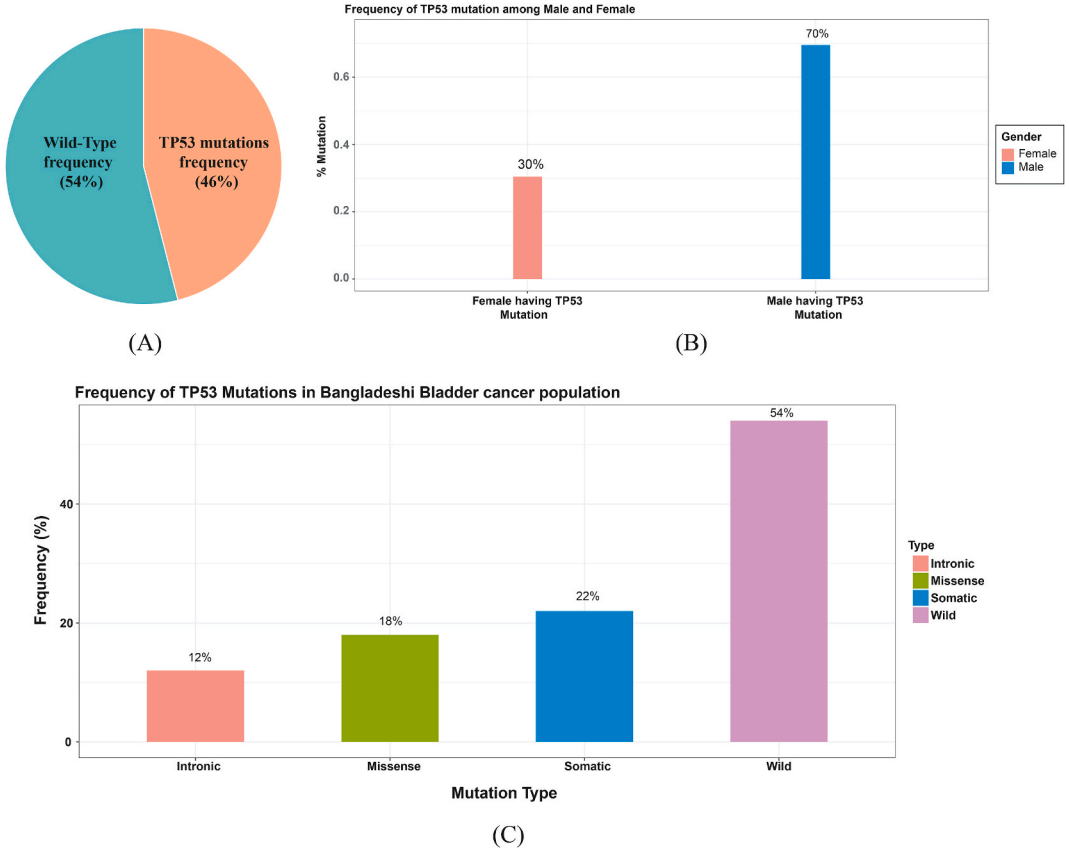

Fig. 3. Frequency of *TP53* mutations in bladder cancer. (A) A pie chart representing the overall proportion of mutations in Bangladeshi bladder cancer patients. (B) A bar plot showing higher mutational frequency in males compared to females. (C) A bar plot representing the frequencies of different types of mutations found from the mutational analysis of exons 5, 7, and 8 of *TP53* in the Bangladeshi bladder cancer population.

Table 2
The mutation position, type, and frequency in exons 5, 7, and 8 in the TP53 gene.

Exon	Genomic Changes	Mutation	Variant Type	Frequency	Count	Global Maf	Total	Grand Total
5	chr17: g.7675216C > G	K132 N	Somatic	4 %	2 in 50	–	6 %	**46 %**
	chr17: g.7675143C > A	V157F	Somatic	2 %	1 in 50	–		
7	chr17: g.7674229C > T	G245D	Somatic	4 %	2 in 50	–	20 %	
	chr17: g.7674252C > G	M237I	Somatic	2 %	1 in 50	–		
	Chr17: 7674109 C > T (rs12947788)	–	Intronic	12 %	6 in 50	0.178		
	Chr17:7674089 T > G (rs12951053)	–	Intronic	4 %	2 in 50	0.178		
8	chr17:7673774 T > A	R2833S	Somatic	6 %	3 in 50	–	24 %	
	chr17: g.7673788G > T	P278T	Somatic	2 %	1 in 50	–		
	chr17: g.7673734G > A	H296Y	Missense	18 %	9 in 50	–		
	chr17: g.7673802C > T	R273H	somatic	2 %	1 in 50	–		

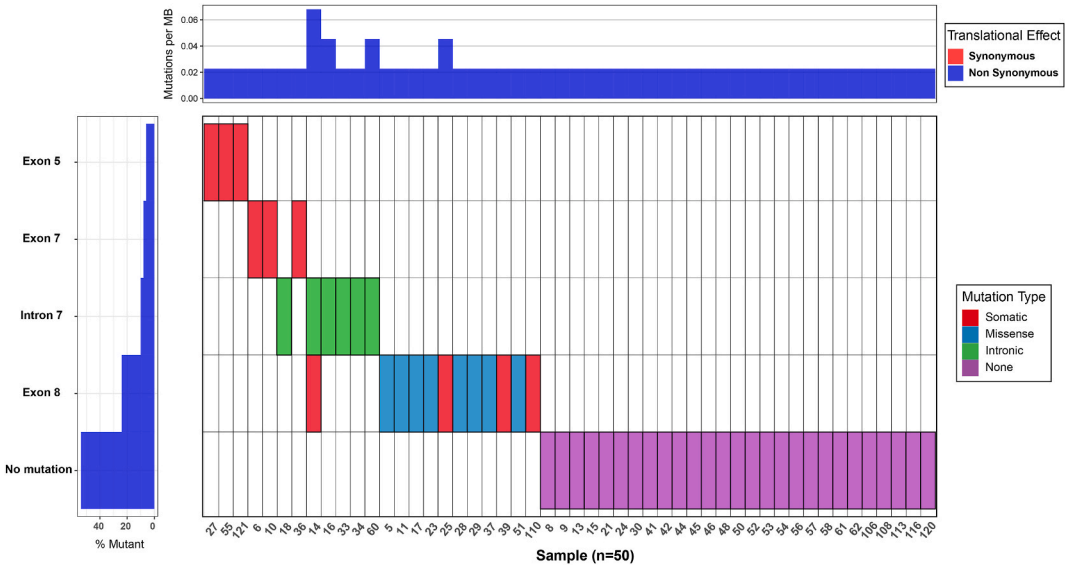

Fig. 4. Graphical representation of bladder cancer patients carrying mutations along with the frequency. This figure provided details about different mutations in patients denoted by different colors along with the frequency of mutations of each exon (left bar plot portion).

3.4. Mutational impact upon the expression of TP53

A total of 46 cDNA, prepared from RNA samples of 46 bladder cancer patients and 15 cDNA prepared from RNA of 15 urine-healthy control, was available for gene expression analysis. To remove any biases from the expression study, control samples were chosen according to the confounding variables of tumor patients' data. There was an equal proportion of males and females in both the control and patient cohorts. There were also no significant differences in age between the two cohorts. This ensured an unbiased expression analysis without concern about any effects from confounding variables (Supplementary Fig. 3).

As *TP53* is frequently mutated in tumor tissue compared to non-cancerous cells, the tumor-specific expression pattern was observed for *TP53* and *TP53*-target gene *BTG2* by taking urine cDNA as control. For this analysis, the ΔCt method was applied which means the expression ΔCt value of *TP53* and *BTG2* genes were normalized by housekeeping reference gene GAPDH. For expression analysis, the ΔCt value represents the inverse of the expression which means a high ΔCt value denotes a low expression value.

Expression analysis between the control and tumor patients showed a statistically significant decrease in the expression of *TP53* in the tumor compared to control samples as tumor samples tend to have a high ΔCt value (Fig. 5A). However, no changes in the expression pattern were observed between somatic mutated and non-mutated or wild-type samples indicating the somatic mutations were not affecting the expression of *TP53* itself (Fig. 5B), and this scenario was expected as the obtained mutations were inactivating mutations and affecting the *TP53* activity.

3.5. Impact of somatic mutations on the expression TP53-target gene BTG2

To observe how the somatic mutations affect the downstream mechanisms that allow cancer progression, the expression pattern of *TP53*-target gene *BTG2* in control, *TP53*-somatic mutant, and *TP53*-nonmutant was observed. The analysis revealed that *BTG2* mean

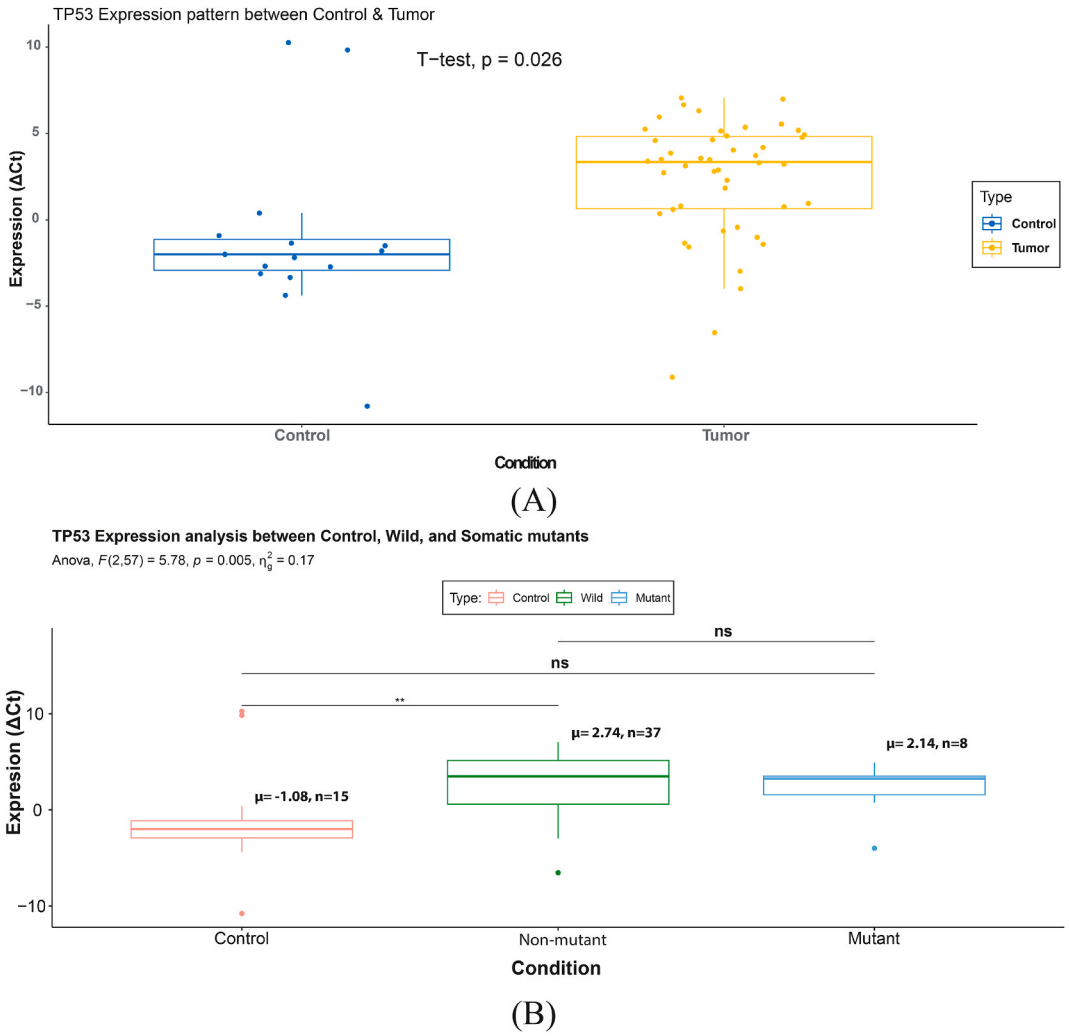

Fig. 5. Boxplot representation of *TP53* expression pattern between (A) Control and bladder cancer patients where an independent T-test was performed giving a p-value <0.05. (B) Expression analysis was conducted between control, somatic-mutant, and non-mutant patients. An Anova test was performed between these three groups giving a p-value <0.05 which indicated a significant difference in means among these groups. No significant difference was observed between mutant and non-mutant groups (p-value >0.05).

expression was lower in tumor tissue compared to control although the result was not significant (p = 0.64) (Fig. 6A). However, in the mutant patients, there was a tendency for high expression of *BTG2* compared to wild-type patients (Mutant ΔCt-median < Wild ΔCt-median) (Fig. 6B). A previously performed study showed that endogenous expression of *BTG2* is associated with poor bladder cancer patient survival and silencing this gene inhibits cell proliferation [11].

Further evidence was observed where *BTG2* expression increased (lower ΔCt value) and *TP53* expression slightly decreased in high-grade patients (Fig. 7) compared to low-grade patients. This increased expression of *BTG2* in high-grade patients supported the significance of *BTG2* in cancer and this also explained the relevance of somatic mutations in cancer progression as the somatic mutant group showed increased *BTG2* expression.

3.6. Impact of H296Y and intronic mutations on cancer

A missense mutation, H296Y, was found within the Bangladeshi bladder cancer population, and there is no report regarding the functional consequences of this mutation. To comprehend, how this mutation was influencing bladder cancer progression the samples were subtyped into two groups, the H296Y-mutant group and the wild-type group, and subsequently, expression analysis was performed between these two groups for *TP53* and *BTG2*. Interestingly, it was observed that the patients carrying this mutation had a tendency to express *TP53* and *BTG2* in higher amounts and as these two genes are tumor suppressors, expressing in high amounts will lead to protection against bladder cancer progression (Fig. 8). Therefore, this mutation, H296Y, may have a protective role against cancer advancement although further study is required to support this observation.

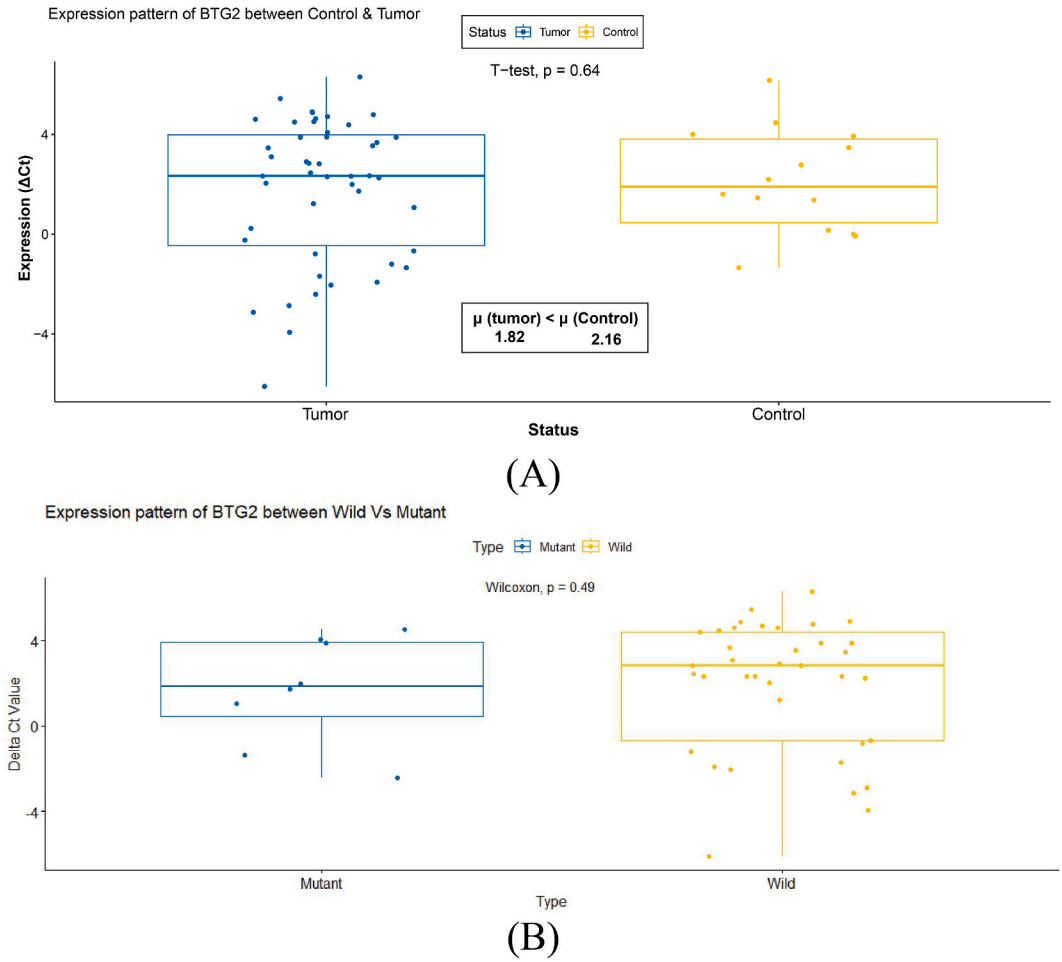

Fig. 6. Boxplot representation of *BTG2* expression compared between (A) tumor-control, and (B) wildtype-mutant patients. Mean *BTG2* expression was lower in patients compared to control (p-value >0.05) while there tends to be a high expression of *BTG2* in the mutant group compared to wild-type patients.

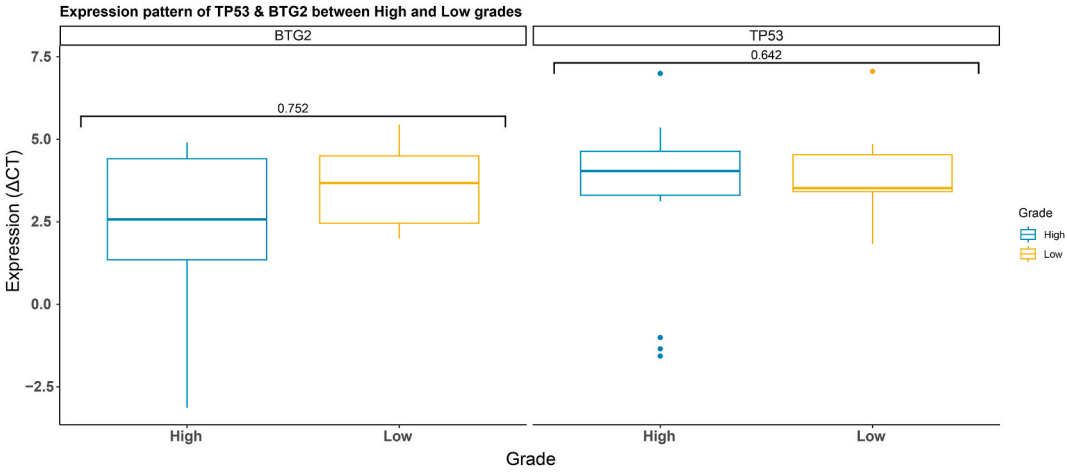

Fig. 7. Expression pattern of (A) *BTG2* and (B) *TP53* in high and low-grade patients. Wilcoxon rank test was performed for individual analysis. A high mean expression of *BTG2* was noticed in high-grade patients with a contrast to lower expression of TP53 in high-grade patients.

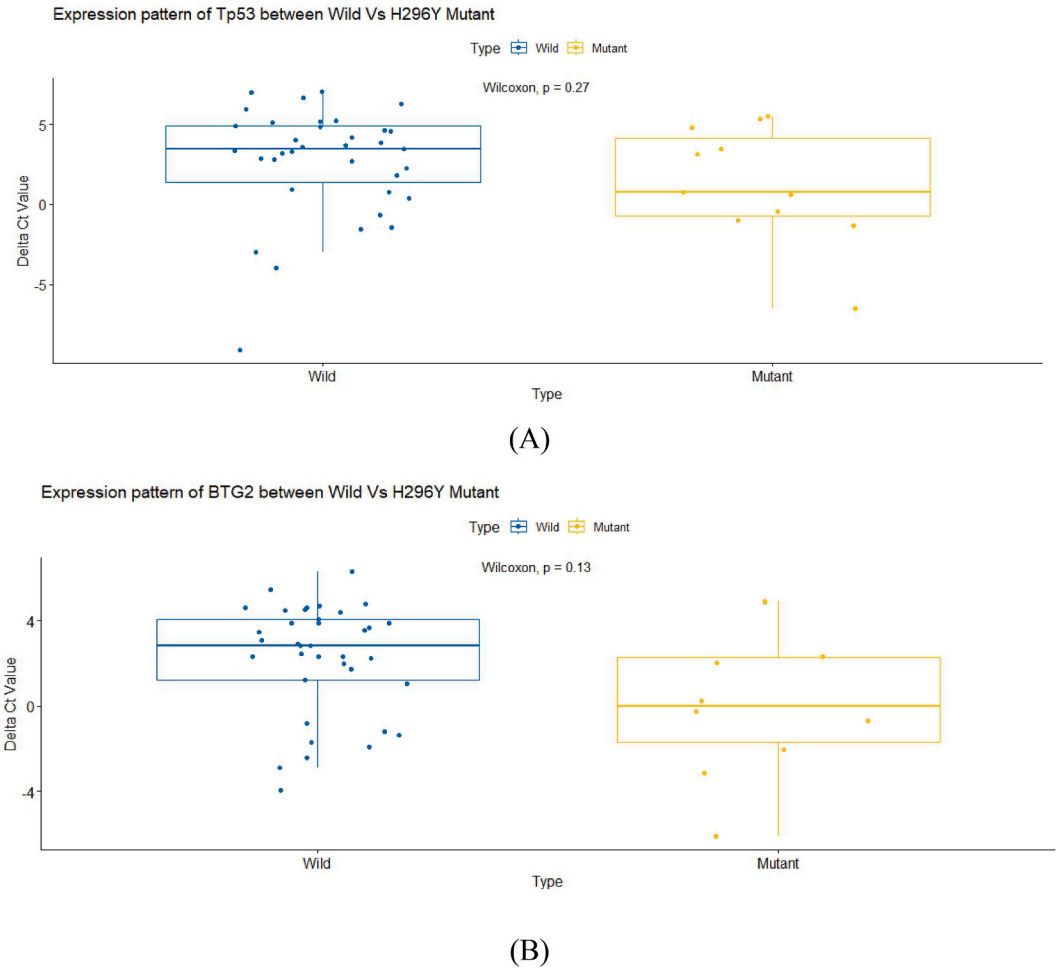

Fig. 8. The expression pattern of (A) *TP53*, and (B) *BTG2* between H296Y-mutant and wild-type bladder cancer patients. Both *TP53* and *BTG2* upregulated in mutant patients. Wilcoxon test was used to observe the significance of the data (p-value <0.05).

In addition, two more intronic variants, rs12947788, and rs12951053, were found in this population which were already reported in the Ensembl database, but these mutations have no impact on regulating the expression of *TP53*. There was no significant difference in *TP53* expression between wild-type and patients carrying the intronic variants (Supplementary Fig. 4).

3.7. Associations of TP53-mutations with bladder cancer patient's survival

To observe how the somatic mutations were impacting the survivability of a patient, survival analysis was performed using available 32 bladder cancer survival data. The analysis of survival data for patients with *TP53* somatic mutations revealed a poor prognosis compared to patients with wild-type *TP53* although the analysis was not statistically significant due to the low number of patient samples (Fig. 9A). This finding was further validated by categorizing TCGA BLCA (bladder carcinoma) data into somatic mutant and wildtype groups based on the observed mutations in this study, which consistently demonstrated a worse prognosis for *TP53*-somatic mutant TCGA patients (Fig. 9B). It was also observed that lower expression of *TP53* in bladder cancer was also associated with poor prognosis in Bangladeshi bladder cancer patients with time. This observation was further confirmed through TCGA survival data analysis, which indicated that patients with lower *TP53* expression had a worse prognosis (Supplementary Fig. 5).

Correspondingly, an association of *TP53*-target gene *BTG2* gene expression with patient survival was also observed and patients with highly expressing *BTG2* showed the worst prognosis for both the Bangladeshi population and TCGA BLCA data (Fig. 10).

Survival analysis was also performed for H296Y-mutant and wildtype patients and better survival was observed for the H296Y-mutant group, supporting the previous observation for its protective role in patients' survival although p-value >0.05 (Supplementary Fig. 6).

Furthermore, survival analysis was performed to determine the strength of intronic variants (rs12951053 and rs12947788) in influencing the survivability of bladder cancer patients and was observed to be associated with the worst prognosis of bladder cancer patients (Supplementary Fig. 7A). This was found consistent with the assessment of the regulatory effect of these variants utilizing the

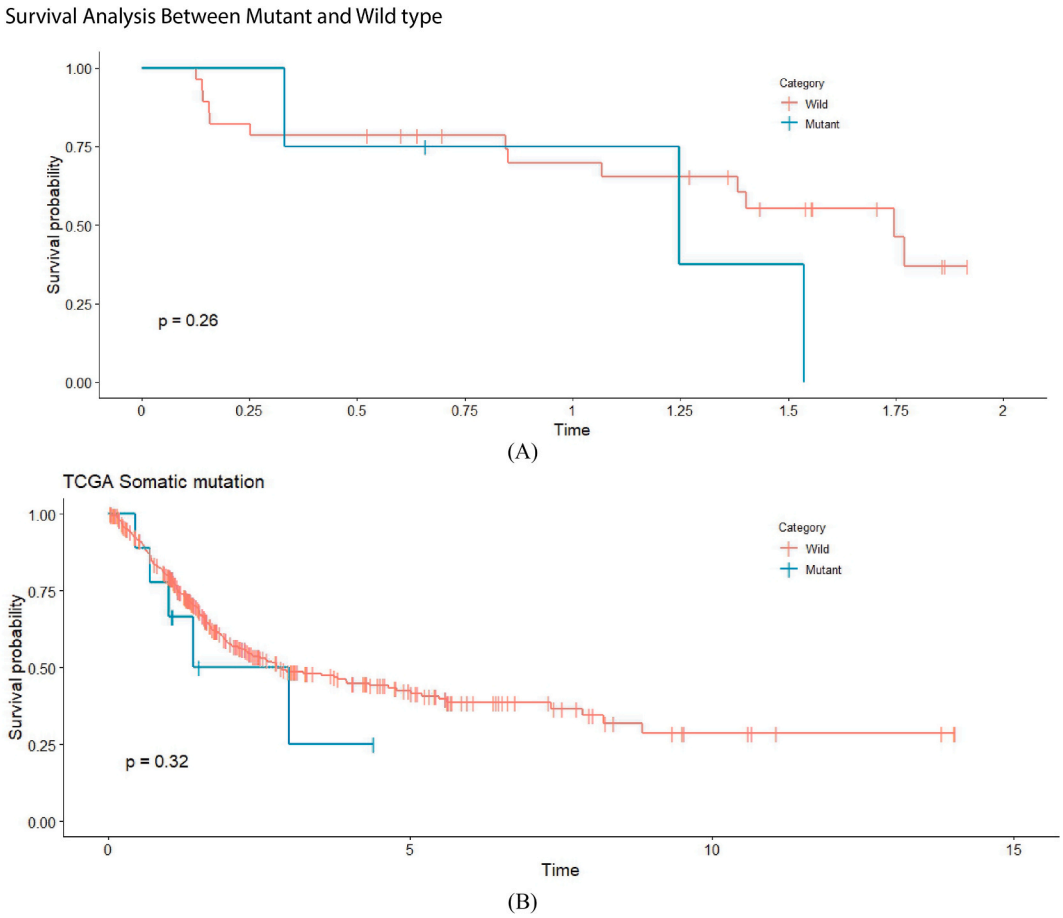

Fig. 9. Survival analysis for bladder cancer patients using the Kaplan-Meier method. Observation of mutational association with (A) Bangladeshi bladder cancer patients, and (B) TCGA bladder cancer data. For both cases, the mutant group showed a poor prognosis compared to the wild-type (p-value >0.05).

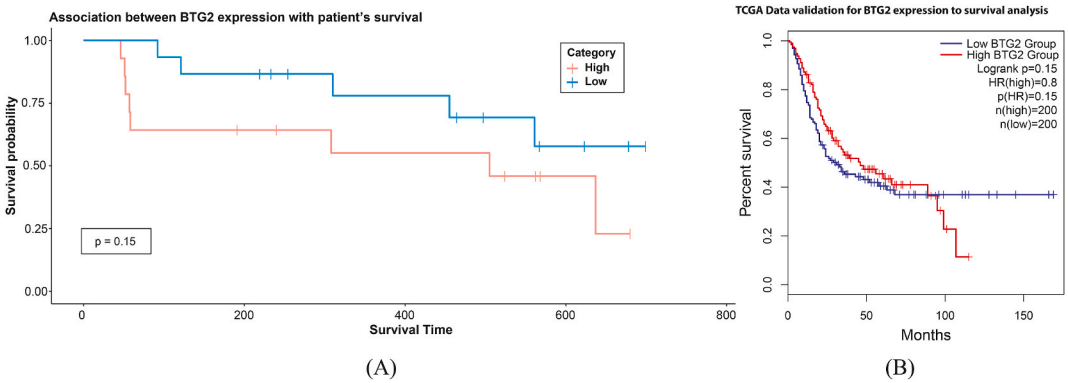

Fig. 10. Association of *TP53* gene expression with the survival status of (A) Bangladeshi bladder cancer patients and (B) TCGA BLCA data. For both cases, patients expressing lower *TP53* were associated with worse prognosis with time.

RegulomeDB web tool where it was found that these variants affect the binding of transcription factors (Supplementary Fig. 7B). Therefore, these intronic variants may have potential implications in bladder cancer progression, and for this further extensive study is required with a large sample size.

3.8. Significance of somatic mutations in cancer progression

TP53 and *BTG2* are two hubs of a large transcription factor network and any disruption in the function of *TP53* ultimately results in an imbalance of such a large network that results in cancer progression (Supplementary Fig. 8A) and somatic mutations were found to be affecting the function of *TP53*.

To further evaluate the influence of somatic mutations on cancer progression, the study calculated the occurrence of high- and low-grade patients in both somatic-mutant and wild-type categories. The findings indicated that among mutant patients, the majority belonged to the high-grade category. Moreover, the frequency of high-grade patients with mutations was higher than those without any somatic mutations (Supplementary Fig. 8B).

3.9. Multivariate analysis of TP53 somatic mutations

A multivariate analysis was performed considering the various confounding factors, including age, gender, smoking, and mutation, to observe the risk assessment capability of the somatic mutations by correlating with the survival status of bladder cancer patients. For this, Cox proportional hazards regression analysis was performed using the survival package, and a forest plot was used to visualize the result of the analysis in R programming language. The analysis revealed that among all the variables mutation had the highest hazard ratio (1.84) although the p-value was greater than 0.05 which can be compensated by increasing the sample size (Supplementary Fig. 9A). This implied that mutation was the best risk assessment tool among all the variables for bladder cancer patients. To validate this result, TCGA BLCA data was utilized for the same purpose, and a similar trend was observed where the mutation had the highest hazard ratio, signifying the importance of these mutations in predicting the risk of bladder cancer patients (Supplementary Fig. 9B).

3.10. Immunological assessment of TP53 somatic mutations

In addition, an immunological assessment of the TP53-somatic mutations was performed using TCGA BLCA data where the data was subtyped into two categories, wild-type and somatic mutant group. For this analysis immune scores for each TCGA BLCA patient were collected from ESTIMATE (https://bioinformatics.mdanderson.org/estimate/) webserver. This immune score represents the abundance of immune cell infiltration in tumors. By comparing the immune scores between the two subtypes, it revealed that the mutant patients tend to have higher immune score compared to the wild type and this implied that the tumor tissue in mutant patients are more abundant with immune cells compared to other patients although the result is not significant (Supplementary Fig. 10A). As a result, these mutant patients may be more susceptible to immune therapy compared to wild-type. Therefore, these somatic mutations can be used in subtyping the bladder cancer population in Bangladesh for a population-specific immune therapy purpose.

3.11. Differential expression and enrichment analysis between somatic mutant and wildtype TCGA patients

Enrichment analysis was performed utilizing the TCGA BLCA data between 9 observed mutated patients containing mutation from the study and 21 wild-type patients bearing no observed mutations. Initially, differential expression analysis was conducted between these two groups using the cBioPortal web tool, and 265 genes were found that were upregulated in the mutated patient based on specific parameter settings (log fold change $> \pm 1$ and adjusted p-value <0.05). These upregulated genes were then subjected to enrichment analysis using the enrichR package in the R programming language. The analysis revealed the enrichment of various immunological hallmark pathways, including inflammatory response, IL-2/STAT5 Signaling, complement, and TNF alpha/beta signaling pathways. The mutated patients are also enriched in biological processes that promote cancer progression, including, epithelial cell proliferation, regulation of cell adhesion, positive regulation of the cellular process, and many other pathways (Supplementary Fig. 10B). Hence, these somatic mutations could potentially drive the advancement of cancer to more advanced stages, while the prevalence of immunological pathways suggests a promising therapeutic approach through immunotherapy for patients with these mutations.

Table 3
Mutational impact on TP53 protein's structure, function, and structure observed by bioinformatic tools.

Mutation	Structural Analysis		Functional Analysis		Stability Asessment		Clinvar classification
	RMSD	TM_Align	SIFT	PON-P2	I-Mutant	Mupro	
R273H	0.212	0.99898	Deleterious	Pathogenic	Decrease	Decrease	Pathogenic
P278T	0.192	0.99895	Deleterious	Pathogenic	Decrease	Decrease	Pathogenic
K132 N	0.187	0.99884	Deleterious	Pathogenic	Decrease	Decrease	Pathogenic
R283S	0.171	0.99922	Deleterious	Pathogenic	Decrease	Decrease	Uncertain significance
V157F	0.175	0.99916	Deleterious	Pathogenic	Decrease	Decrease	Pathogenic
G245D	0.166	0.999	Deleterious	Pathogenic	Decrease	Decrease	Pathogenic
M237I	0.153	0.99929	Deleterious	Pathogenic	Decrease	Decrease	Pathogenic
H296Y	0.19	0.99889	Tolerated	Non-Pathogenic	Increase	Increase	Likely Benign

3.12. Impact of mutations in TP53 protein structure, function, and stability

For validating the influence of mutations on protein structure, function, and stability was assessed using computational tools. The TP53 protein structure was predicted for each coding mutation along with the wild-type TP53 using the Swiss model and refined using the GalaxyRefine tool. All the predicted models were evaluated by Ramachandran plot and all models have more than 90 % residues in the favored regions. Then, the RMSD and TM align score was calculated for each mutated model. All showed a score deviation from 0 for RMSD and 1 for TM-align score, depicting the alteration in the structure from wild-type. Furthermore, functional analysis was performed using SIFT, and PON-P2, where all the somatic mutations disclosed pathogenic characteristics and the missense H296Y mutation showed a non-pathogenic behavior. This aligns with the observation where H296Y mutated patients showed better survival and somatic mutant patients had poor prognoses. Clinvar assessment also supported these observations which dictates the clinical relevance of the mutations. I-Mutant and MUpro servers were used to observe the effect of mutations on protein stability and all somatic mutations decreased the *TP53* protein's stability whereas H296Y increased the protein's stability (Table 3). This explained the protective role of H296Y. All the somatic mutations were also observed in the cosmic database and found to be affecting multiple regulatory pathways including the MAPK signaling pathway, Androgen Receptor Signaling Pathway, cell cycle, apoptosis, and many others. A graphical representation of mutational protein structures was visualized in Supplementary Fig. 11.

4. Discussion

The diagnosis of bladder cancer requires several recurring invasive procedures, such as cystoscopy. Researchers are therefore developing other means, noninvasive and cost-effective, for diagnosing bladder cancer because of the disease's high somatic mutation load. In the majority of cases, the *TP53* function is deactivated due to mutations in the *TP53* gene that frequently occur in bladder cancer [29]. *TP53* mutations, very common in bladder cancer, mainly occur in muscle-invasive cases. A result of the deactivation of *TP53* function in the majority is the driving of disease progression, affecting prognosis and therapy. These mutations initiate and advance cancer, with an increase in metastasis and changing responses to chemotherapy [16]. Somatic mutations, such as *TP53*, give diagnostic markers simple enough to be detected by standard genetic techniques and may revolutionize bladder cancer diagnostics and treatment.

This novel study solely focused on investigating *TP53* mutations alongside gene expression and clinical prognosis to understand the molecular mechanisms underlying cancer progression in the Bangladeshi bladder cancer population. This integrated approach helps researchers and clinicians identify specific genetic alterations, their impact on gene expression patterns, and how these factors collectively influence the clinical outcomes and prognoses of patients with bladder cancers. Such studies are pivotal for developing targeted therapies and personalized treatment strategies, ultimately improving patient care and outcomes in the field of oncology.

The most mutation-prone exons (5, 7, & 8) of *TP53* were considered for Sanger sequencing and mutation analysis in Bangladeshi bladder cancer patients. *TP53* is a regulator of a large transcription network system and therefore, any disturbance in the function of *TP53* may lead to abnormal expression dynamics of many cancer-associated genes, including BTG2. For that, the expression dynamicity of *TP53* and *BTG2* was observed to comprehend the impact of *TP53* mutations. The mutation analysis revealed an overall 46 % mutation frequency (23 patients out of 50), which included somatic, missense, and intronic variants which were found to be consistent with other reports [30]. Among them, 11 patients carried 7 somatic mutations (22 %) and 9 patients carried a missense mutation (H296Y). *TP53* expression was lower in cancer patients compared to healthy control but, no significant difference in somatic mutated and wild-type patients. Survival analysis showed that these somatic mutations were associated with the worst prognosis and this result was found consistent with TCGA BLCA survival analysis. The identified somatic mutations primarily occur in the DNA binding domain of the TP53 protein.

These mutations do not directly impact the expression of *TP53* itself. Instead, they modulate the binding of *TP53* to its specific binding sites within the DNA of *TP53* target genes. To ensure this, *TP53*-target gene *BTG2* expression was observed which is down-regulated in tumor tissue compared to control, but the mutated samples tend to have high expression of *BTG2*. This scenario was also found to be consistent with a previously performed study where the authors showed *BTG2* silencing leads to inhibition of cellular growth, proliferation, and migration in bladder cancer cell lines, although the exact mechanism is unknown [11]. The survival analysis also supported this statement by revealing the worst prognosis with high expression of *BTG2*. As *TP53*-mutated patients showed the worst prognosis with Bangladeshi bladder cancer patients' survival and led to increased expression of *BTG2*, it can therefore be said that these somatic mutations are supportive of cancer progression by hampering downstream mechanisms that promote tumor formation, and these observations were also found consistent with previous studies [31].

When TP53 is inactivated or mutated in bladder cancer, it can lead to uncontrolled cell growth and the formation of tumors that are more difficult to treat. In these cases, cancer cells are less responsive to therapies such as chemotherapy and radiation, making it challenging to manage the disease effectively. Patients with bladder cancer and *TP53* inactivation typically have a worse prognosis compared to those with intact *TP53* function. The inactivation of *TP53* is associated with higher tumor grade, muscle invasion, and metastasis, all of which contribute to poor survival rates in affected individuals [32].

The precise consequences of the H296Y missense mutation remain undocumented. To shed light on its implications, this study conducted essential observations, offering partial insights into the nature of this mutation. Strikingly, survival analysis of patients carrying the H296Y mutation revealed an overall improved prognosis. Additionally, these patients exhibited elevated expression levels of both *TP53* and *BTG2* compared to their wild-type counterparts. This increase supports the notion of impeding cancer progression, given that both *TP53* and *BTG2* are tumor suppressor genes.

Two intronic variants (rs12947788 & rs12951053) were discovered in 6 bladder cancer patients and the RegulomDB server

revealed these introns have regulatory effects and can hamper transcription factor binding and distort motifs. The negative effects of these introns were also supported by the association with poor survival of Bangladeshi bladder cancer patients. Previous studies reported a risk association between rs12947788 and colorectal and pancreatic cancers. Therefore, it is required to signify the importance of this variant in bladder cancer [33]. It is also proposed that rs12951053 along with other *TP53* somatic mutations associated with genomic instability in breast and esophageal cancer [34]. So, whether this hypothesis extends to bladder cancer or not also needs to be examined.

Following this, the multivariate analysis demonstrated the superior predictive ability of mutations when compared to other potential confounding factors. Additionally, the heightened prevalence of high-grade patients within the somatic mutant groups provided collective evidence supporting the significant impact of *TP53* mutations on the progression to more advanced stages of cancer. In addition, another attempt was made to justify the poor clinical implication of these mutations in bladder cancer patients by performing an enrichment analysis between mutated and non-mutated patients utilizing TCGA bladder cancer data which disclosed that immunological pathways along with critical cancerous biological processes were enriched in mutated patients and this was found consistent with the higher immune scores for mutated patients. Consequently, this result proposed that the patients can be subtyped based on *TP53* mutations and immune therapy could be notably effective for patients with *TP53* mutations. Alterations in structure, functions, and stability due to the coding mutation of *TP53* were analyzed through computational analyses and these analyses revealed potential adverse effects on structure, functions, and stability, except for the H296Y missense mutation, which was determined to be non-pathogenic and even enhanced the protein's stability. These findings align with the earlier observations for the H296Y mutation. Even though potential supportive observations were found in this research work, further study will be performed by increasing the sample size with increased survival data, which is a major limitation of this study, upon recruiting enough bladder cancer patient population.

Some regulators control the activity of *TP53*, including *MDM2*. This protein regulates the *TP53* pathway through several mechanisms, including direct interaction with *P53,* export of *P53* out of the nucleus, and promote *P53* degradation [35]. *MDM2* is often over-expressed in cancer and this hampers the activity of *TP53* [36]. The observed mutations were mostly present in the DNA binding domain and so the transcriptional activity of *TP53* was hampered as the downstream gene *BTG2* where dysregulated expression was observed. Even if *MDM2* does decrease the activity of *TP53*, the presence of these mutations may act additively in hampering the transcriptional activity of *TP53* that altogether promotes cancer progression. Also, there are *MDM2* effect-independent P53 pathways as well where these mutations may exert their effect much more strongly [37]. Therefore, this study independently signified the impact of the mutation on the activity of *TP53*.

The unique nature of this study was attributing the mutations of the TP53 gene, the gene expression patterns, and clinical outcomes specifically in Bangladeshi patients with bladder cancer. Through this focused work, the research pinpointed this population's mutation profiles and their role in individual prognosis. This knowledge gave first-hand data which were analyzed to deliver personalized treatment options depending upon the unique genetic traits of Bangladeshi bladder cancer patients. Through specific targeted research and followed by absolute confirmation and comparison of Bangladeshi data to global data, this study is an advanced initiative to understand bladder cancer genetics and personalized medicine in the Bangladeshi context.

In conclusion, this study explored an important landscape of cancer biology by examining the mutational impact on the tumor suppressor gene, *TP53*, and identified potential mutations that were associated with the survival of bladder cancer patients in Bangladesh following other bladder cancer reports. The study rigorously analyzed the data including mutation and expression considering TCGA BLCA data as validation material. Altogether, these mutations prevalent in the Bangladeshi bladder cancer population may serve as prominent drivers, significantly impacting *TP53* function and mechanisms. Therefore, this study will lay a foundation for further large-scale molecular studies and provide support to shape a therapeutic model for early diagnosis and treatment strategy for the bladder cancer population in Bangladesh.

Data availability

All Data have been provided with the manuscript.

CRediT authorship contribution statement

Dipankor Chatterjee: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Shabiha Afroj Heeamoni:** Data curation, Investigation, Methodology, Validation. **Tamanna Sultana:** Data curation, Investigation, Validation, Visualization. **Sadia Islam Mou:** Data curation, Investigation, Validation, Visualization. **Munshi Akid Mostofa:** Formal analysis, Investigation, Methodology, Resources, Visualization. **Md Akmal Hossain:** Data curation, Resources, Validation. **Md Ismail Hosen:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Md Omar Faruk:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31286.

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