Clinical Significance of TP53, PAX5, and JAK2 gene mutation in Pediatric Acute Lymphoblastic Leukemia

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Abstract

Background: Pediatric acute lymphoblastic leukemia (ALL) is the most common childhood cancer worldwide. Developed countries have a 90% 5-year overall survival rate with proper treatment, while LMICs have a poor rate of around 30-50%. AIM: The research aims to identify mutations in frequently mutated genes’ hotspot regions to design appropriate treatment plans based on patients’ somatic makeup. Methods: Sanger sequencing was conducted on TP53, PAX5, and JAK2 gene hotspot regions in 60 Patients with ALL diagnosed with acute lymphoblastic leukemia, categorized into B-ALL and T-ALL subtypes. Results: The exon mutation rate was 8.33%. The mutation frequency for PAX5 was 5%, while for TP53, it was 3.33%. New mutations found in TP53 and PAX5 genes intron region. None of these mutations was found significant to have a poor prognosis either on the whole cohort or chemotherapy recipient patients. Among the mutated samples, Chr17:7674089 (A-C) and Chr17:7674109 (G-A) were found to have a worse prognosis in patients diagnosed with T-ALL. Chemotherapy treatment response is significant with p = 0.011, and there was a linkage between chemotherapy response and the overall mutation in chemotherapy patients (p=0.0013). The TP53 mutation in chemotherapy patients is related to poor survival (p=0.001) rather than the PAX5 mutation (p=0.087). Conclusion: TP53 gene mutation is associated with poor chemotherapy response, and subtypes specific study is required for the precise treatment plan for Bangladeshi pediatric patients with ALL.
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ABBREVIATIONS:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ALL</td>
<td>Acute Lymphoblastic Leukemia</td>
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<tr>
<td>B-ALL</td>
<td>B- cell acute lymphoblastic leukemia</td>
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<tr>
<td>T-ALL</td>
<td>T- cell acute lymphoblastic leukemia</td>
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<tr>
<td>HC</td>
<td>Higher Income Country</td>
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<td>Lower Middle-income Country</td>
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<td>TP53</td>
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<td>PAX5</td>
<td>Paired box 5</td>
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<td>JAK2</td>
<td>Janus Kinase 2</td>
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ABSTRACT:

Background: Pediatric acute lymphoblastic leukemia (ALL) is the most common childhood cancer worldwide. Developed countries have a 90% 5-year overall survival rate with proper treatment, while LMICs have a poor rate of around 30-50%.

Aim: The research aims to identify mutations in frequently mutated genes’ hotspot regions to design appropriate treatment plans based on patients’ somatic makeup.

Methods: Sanger sequencing was conducted on TP53, PAX5, and JAK2 gene hotspot regions in 60 Patients with ALL diagnosed with acute lymphoblastic leukemia, categorized into B-ALL and T-ALL subtypes.

Results: The exon mutation rate was 8.33%. The mutation frequency for PAX5 was 5%, while for TP53, it was 3.33%. New mutations found in TP53 and PAX5 genes intron region. None of these mutations was found significant to have a poor prognosis either on the whole cohort or chemotherapy recipient patients. Among the mutated samples, Chr17:7674089 (A-C) and Chr17:7674109 (G-A) were found to have a worse prognosis in patients diagnosed with T-ALL. Chemotherapy treatment response is significant with \( p = 0.011 \), and there was a linkage between chemotherapy response and the overall mutation in chemotherapy patients \( (p=0.0013) \). The TP53 mutation in chemotherapy patients is related to poor survival \( (p=0.001) \) rather than the PAX5 mutation \( (p=0.087) \).

Conclusion: TP53 gene mutation is associated with poor chemotherapy response, and subtypes specific study is required for the precise treatment plan for Bangladeshi pediatric patients with ALL.

MAIN TEXT

1. Introduction:

Childhood cancer is cancer that develops in adolescents. It is uncommon and emerges between birth and 15 years, posing a significant public health risk. According to the World Health Organization (WHO), an estimated 400,000 additional cancer cases among children under 15 will occur worldwide in 2021. This accounts for around 1.3% of all new cancer cases diagnosed that year.
Leukemia shares a significant portion of pediatric cancer. Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer worldwide. Cancer occurs for many reasons, according to the hallmarks of cancer. Mutation in the vital signaling pathways associated with cellular growth, progression, survival, and apoptosis will result in cancer. Extensive research has shed light on the genetic changes that underpin the development and progression of pediatric ALL throughout the years, leading to the identification of many gene variants related to the illness. Mutations in the TP53, PAX5, and JAK2 genes have emerged as prominent genetic aberrations with severe clinical consequences in pediatric ALL.

TP53 gene encodes the tumor protein p53, which is a crucial regulator of cell cycle progression, DNA repair, and death. TP53 mutations are well-known oncogenesis drivers in various cancers, including ALL. TP53 mutations are more common in high-risk pediatric patients with ALL and are linked with poor treatment outcomes and higher recurrence rates. According to several research, the prevalence of TP53 mutations in pediatric ALL ranges from 3% to 10%. It is crucial to remember that high-risk categories, such as individuals with relapsed or refractory cancer or those with specific cytogenetic abnormalities, may have a higher prevalence of TP53 changes. These mutations provide medication resistance and impede the apoptotic response, allowing leukemic cells to avoid cell death processes. As a result, individuals with TP53 mutations frequently fail to respond to therapy and have a much worse overall survival rate. Early diagnosis of TP53 mutations is critical for risk assessment and personalized treatment regimens in these high-risk individuals to enhance clinical outcomes.

PAX5 gene encodes a transcription factor essential for B-cell development. PAX5 mutations can interfere with B-cell development, causing issues in pediatric ALL cases, especially BCP subtype ones. Reports suggest 20-40% of disease is due to PAX5 mutations. PAX5 mutations, including gene deletions, point mutations, and gene fusions, can significantly affect treatment outcomes and disease risk. They can interrupt regular gene expression and B-cell maturation, leading to abnormal fusion proteins or lost PAX5 function. Addressing PAX5 mutations is crucial due to their clinical significance, as they can increase recurrence likelihood and negatively impact treatment response. Furthermore, PAX5 mutations frequently co-occur with other genetic disorders, such as CRLF2 gene rearrangements, resulting in a more severe disease phenotype.

JAK2 is a tyrosine kinase that signals for hematopoiesis. Recent research found that JAK2 gene mutations V617F and G683R in pediatric ALL are less common than in myeloproliferative neoplasms. JAK2 mutations have a relatively low reported prevalence in pediatric ALL, ranging from 1-2%. JAK2 mutations are more common in BCP-Patients with ALL and often co-occur with CRLF2 rearrangements. JAK2 mutations cause abnormal JAK-STAT signaling, which promotes cell proliferation and survival. JAK2 mutations have been linked to a greater risk of recurrence and a worse response to treatment in pediatric ALL, underlining their clinical importance. For individuals with JAK2 mutations, targeting the JAK-STAT system with particular inhibitors is a viable therapeutic option.

Studying these gene mutations in new patients with ALL and tracking their response to chemotherapy can improve healthcare and reduce disparities between higher-income (HC) and lower-middle-income (LMIC) countries. A recent study showed more than a 45% disparity in acute lymphoblastic leukemia has demonstrated between HC and LMIC. Therefore, screening the newly diagnosed patient with ALL with the most frequent mutated gene in this study could better establish the possibilities of these mutations’ clinical effects on the overall survival and the chemotherapy response.

2. Methods:

2.1 Patients and ethical statement:

60 Patients with ALL (13) years old were taken for this study. The blood samples were collected through venipuncture and stored in K2-EDTA tubes from the Pediatric Hematology and Oncology Department of Dhaka Medical College Hospital between 2020 and 2022. Blood samples were preserved at -20 within 24 hours of collection.

2.2 Genomic DNA extraction:
Genomic DNA was obtained from BM (bone marrow) or WB (whole peripheral blood) samples at the first diagnosis. DNA extraction was performed using the QIAamp DNA Mini Kit (Cat. # 51304, Qiagen, Hilden, Germany). NanoDrop™ One (Thermo Fisher Scientific, USA) was used to quantify and qualify extracted DNA. Working dilutions of 10ng/ul of each sample DNA were produced and kept at -20°C.

2.3 Target gene amplification:

2.3.1 Target gene selection:

We carried out a comprehensive analysis, which involved conducting a thorough literature review, utilizing the cBioPortal (https://www.cbioportal.org/), and thoroughly examining the COSMIC (https://cancer.sanger.ac.uk/cosmic) database. This analysis identified the hotspot region of the most commonly mutated gene in pediatric acute lymphoblastic leukemia. In this study, the most frequently mutated and selected genes associated with pediatric ALL were TP53, PAX5, and JAK2.

2.3.2 Primer designing:

Primer pairs were designed against the hotspot region of the selected gene using the GenBank reference sequence of TP53 (NG_017013.2), PAX5 (NG_033894.1), and JAK2 (NG_009904.1) genes using the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) from NCBI. Table 1 provides the primer sequence, PCR product size of primers, and the optimum temperature for the PCR reaction needed to amplify the TP53, PAX5, and JAK2 genes. Following Table 1, the Ensemble Transcript IDs of three important genes TP53, PAX5, and JAK2 were ENST00000269305, ENST00000358127, and ENST00000381652, respectively.

Table 1: Primer sequences of targeted gene (TP53, PAX5, and JAK2) region with their product sizes (bp) and optimum temperature (Tm) from gradient PCR.

2.3.3 PCR (Polymerase chain reaction) optimization:

For PCR reaction DreamTaq Green Master Mix and DreamTaq PCR Master Mix (Cat. # K1081 and Cat # K1071, Thermo Scientific Corp., USA) were used using the Biometra Tadvanced 96S (Analytik Jena) thermal cycler.

2.3.4 Gel electrophoresis:

After amplification, 5μl of PCR products were run in 2% agarose gel electrophoresis with the “Quick-Load® Purple 100 bp DNA Ladder” (New England BioLabs Inc., UK.), “Thermo Scientific™ DNA gel loading dye 6X” (ThermoFisher Scientific Ltd, USA), and “SYBR Safe DNA Gel Stain” (Invitrogen, ThermoFisher Scientific Ltd, USA). DNA bands were visualized upon UV illumination to ensure the presence of the expected size of DNA amplicons, and an agarose gel image was captured by Alphalmager MINI digital imaging system.

2.4 Sequencing data analysis:

The amplicon PCR products were purified and subjected to capillary electrophoresis for sequencing on the AB135000 Genetic Analyzer. The obtained sequence files were examined using Geneious Prime 2023 with the respective NCBI Reference Sequence to identify any changes in the coding exons or other variants.

2.5 Survival analysis:

For statistical analysis, version 4.2.2 of the R programming language was utilized. The Kaplan Meier Curve, Log-rank tests were performed using this language. P value below 0.05 was considered statistically significant.

3. Results:

3.1 Clinicopathological features:

Pediatric acute lymphoblastic leukemia (ALL) patients’ clinical and hematological information was collected with their consent, and those characteristics are given in Table 2.
Table 2: Clinicopathological characteristics of pediatric acute lymphoblastic leukemia patients in this study.

3.2 Target gene amplification:

Hotspot mutation sites of \( TP53 \), \( PAX5 \), and \( JAK2 \) genes, according to Table 2, were PCR amplified, and the desired band was observed for each gene performed by gel electrophoresis.

3.3 Mutation analysis of the TP53 gene:

3.3.1 Exon region mutation:

Mutation analysis of the exons 5, 7, and 8 of the \( TP53 \) gene was carried out for patients with ALL included in this study (Table S1). Sanger sequencing indicated that \( TP53 \) mutations were detected in 2 of 60 ALL cases, for a mutation frequency of 3.33% (Fig. 1AI). Only B-ALL (4.08%) had \( TP53 \) mutations (Fig. 1AII). There was 1 case (ID 29) of C AG-A AG where codon 167 was changed from glutamine to lysine (Gln167Lys), and one case of a novel mutation observed in codon 289, Leu289Phe C TC-T TC in ID 26. Residues mutated were determined by ensuring a minimum base quality of 20 (Fig. 2B).

Figure 1: Exon mutation of the \( TP53 \) gene in pediatric acute lymphoblastic leukemia. A) Pie chart showing the percentage of mutation among the samples (I), whereas II depicted the percentage of wild-type and mutant-type \( TP53 \) genes in B-ALL. B) Chromatograms showing mutation, the red circle marked the mutation position. (I) Gln167Lys (C AG-A AG) substitution, and (II) Leu289Phe (C TC-T TC) substitution. C) Lollipop chart containing the mutation sites of the \( TP53 \) gene in the samples. D) Mutational effects on the overall survival of the whole cohort between wild-type and mutant-type in \( TP53 \) mutation were statistically insignificant (p-value 0.8). E) Mutational effects of \( TP53 \) mutation on the overall survival of the chemotherapy recipient patients were statistically significant (p-value 0.001) and showed poor prognosis in chemotherapy mutant patients.

Table S1: Mutant patients (exon region) of pediatric ALL with their features.

3.3.2 Intron region mutation:

After analysis of the PCR amplicon of the \( TP53 \) gene, two novel mutations were observed in the intronic region Chr17:7674089 and Chr17:7674109 (Table S2). For Chr17:7674089, adenine (A) mutated with cytosine (C). In contrast, for Chr17:7674089, guanine (G) mutated with adenine (A). Mutated residues are attributed to having a base quality of 0.20. 12 patients were mutated (A-C) out of 60 in the intronic site (Chr17:7674089). Fig S1A shows the percentage of mutated cases from the perspective of the whole cohort, B-ALL, and T-ALL. The Chromatogram of the mutated sample presenting in Fig. S1B. Fig. S1C-E displays the distribution of gender, subtypes, and chemotherapy recipient patients among the mutated samples. Mutational effects were checked in both whole cohorts and chemotherapy recipient patients; both were found statistically insignificant, with a p-value of 0.096 (Fig. S1F) and a p-value of 0.34 (Fig. S1G), respectively.

Figure S1:Chr17:7674089 A-C intronic mutation of \( TP53 \) gene in pediatric ALL. A) Pie chart showing the percentage of the whole cohort of the Chr17:7674089 A-C intronic \( TP53 \) gene mutation percentage in Patients with ALL (I), whereas II and III depicted the percentage of wild-type and mutant-type \( TP53 \) gene in B-ALL and T-ALL. B) Chromatograms showing a mutation in Chr17:7674089 (A-C) of \( TP53 \) gene; C-E) Donut chart displaying the percentage of patients based on gender, subtypes, and treatment in mutated Patients with ALL. F-G) Knplot represents the mutational effects on overall survival of the whole cohort (F) and chemotherapy recipient (G) between wild-type and mutant-type in \( TP53 \) were statistically insignificant.

Table S2: Mutant patients (Intron region) of pediatric ALL with their features.

On the other hand, there were 5 mutated cases among the 60 pediatric Patients with ALL (Fig. S2A). Fig. S2B shows the chromatograms of Chr17:7674109 (G-A) of the \( TP53 \) gene. Fig. S2C-E illustrates the distribution of mutated samples among patients based on their gender, subtypes, and treatment history.
None of the whole cohort patients and chemotherapy recipient patients shows statistical significance due to the mutation occurrence at this mutation site with a p-value of 0.42 and 0.99, respectively (Fig. S2F-G).

**Figure S2:** Chr17:7674109 G-A intronic mutation of TP53 gene in pediatric ALL. A) Pie chart showing the percentage of the whole cohort of the Chr17:7674109 G-A intronic TP53 gene mutation percentage in Patients with ALL (I), whereas II and III depicted the percentage of wild-type and mutant-type TP53 gene in B-ALL and T-ALL. B) Chromatograms showing a mutation in Chr17:7674109 (G-A) of TP53 gene; C-E) Donut chart displaying the percentage of patients based on gender, subtypes, and treatment in mutated Patients with ALL. F-G) Kmplot represents the mutational effects on overall survival of the whole cohort between wild-type and mutant-type in TP53 gene statistically insignificant (p-value 0.42) and the mutational effects on overall survival of chemotherapy recipients (G) between wild-type and mutant-type of TP53 gene also statistically insignificant with a p-value of 0.99.

### 3.4 Mutation analysis of the PAX5 gene:

#### 3.4.1 Exon region mutation:

Mutation analysis of the exons 2 and 3 of the PAX5 gene was carried out for patients with ALL included in this study. Sanger sequencing revealed that PAX5 mutations were present in 3 out of 60 ALL cases constituting the mutation frequency of 5% (Fig. 2A). These mutated residues are considered due to having [?] 20 base quality.

In the PAX5 gene mutation, one patient (ID 39) showed a mutation in the hotspot mutation site in the PAX5 gene exon 2 (codon 26), which was changed from GT T-GG T, resulting in Val26Gly substitution. In addition, two patients (ID 28, 51) had shown a novel mutation in PAX5 gene exon 3 codons 84 and 91, respectively, which were altered to GG A - GA A and G CC-C CC, resulting in Gly84Glu and Ala91Pro substitution, respectively (Fig. 2B).

**Figure 2:** Exon mutation of the PAX5 gene in pediatric acute lymphoblastic leukemia. A) Pie chart showing the percentage of mutation among the samples (I), whereas II and III depicted the percentage of wild-type and mutant-type PAX5 genes in B-ALL and T-ALL. B) Chromatograms showing mutation, the red circle marked the mutation position. (I) Val26Gly (GT T -GG T) substitution, (II) Gly84Glu (GG A-GA A) substitution, and (III) Ala91Pro (G CC - C CC) substitution. C) Lollipop chart containing the mutation sites on the PAX5 genes in the samples. D) Kmplot represents the mutational effects on the overall survival of the whole cohort between wild-type and mutant-type in PAX5 mutation with a p-value of 0.14 which was statistically insignificant. E) Kmplot represents the mutational effects on overall survival of chemotherapy recipients between wild-type and mutant-type in PAX5 gene also found statistically insignificant with a p-value of 0.087.

#### 3.4.2 Intronic region mutation:

After analysis of the PCR amplicon of the PAX5 gene, a novel mutation is observed in the intronic region (Chr09:37020625); on this site, Adenine (A) mutated with cytosine (C). Mutated residues are attributed to having a base quality of [?] 20. A total of 39 patients were found mutated among 60 Patients with ALL. Male patients are frequently mutated compared to females (Table S2). Fig. S2A depicts the percentage of mutation occurrence. Chemotherapy recipient patients and B-ALL were also seen as frequently mutated compared to non-chemotherapy and T-Patients with ALL (Table S2). Fig. S3B-D provides an overview of the distribution of mutated samples in patients based on their gender, subtypes, and treatment history. There is no statistical significance in the occurrence of mutations at this mutation site for both the whole cohort (Fig. S3E) and chemotherapy recipients (Fig. S3F) patients. The p-values for these groups are 0.99 and 0.46, respectively.

**Figure S3:** Chr09:37020625 A-C intronic mutation of PAX5 gene in pediatric ALL. A) Pie chart showing the percentage of the whole cohort of the Chr9:37020625 A-C intronic PAX5 gene mutation percentage in Patients with ALL; B - D) Donut chart displaying the percentage of patients based on gender, subtypes, and treatment in mutated Patients with ALL. E) Kmplot represents the mutational effects on
overall survival of the whole cohort between wild-type and mutant-type in PAX5 mutation is statistically insignificant with a p-value of 0.99; F) Kmplot represents the mutational effects on overall survival on chemotherapy recipient between wild-type and mutant-type in PAX5 mutation with a p-value of 0.46 which is also statistically insignificant.

3.5 Mutation analysis of the JAK2 gene:

Mutation analysis of exon 16 (hotspot region for patients with ALL) of the JAK2 gene was performed for patients with ALL included in this study through Geneious Prime 2023. Sanger sequencing revealed no mutation found in either exon or intronic region regarding the base quality of [?]20.

3.6 Survival analysis:

Overall survival (OS) between wild and mutant types in TP53 and PAX5 gene exon and intron region mutation in the whole cohorts and chemotherapy recipient patients were measured. OS curve was also measured based on the treatment response, gender, and cancer subtypes for both exon and intron mutation. The total surviving mean follow-up time was 6.9 months (0.1-22 months). Chemotherapy treatment helps recover the pediatric Patients with ALL as we observed a significant difference between chemotherapy and no chemotherapy recipient patients having an overall survival with a p-value of 0.011 (Fig. 3B ). Kaplan – Meier OS curve shows (Fig. 3CI ) a tendency to poor prognosis in females in whole cohorts though it was not found statistically significant (p-value 0.071). In chemotherapy response, the females were supposed to be worse prognoses than men. However, statistical significance was not found regarding a p-value of 0.068 (Fig. 3DI ). No significance was found regarding cancer subtypes in survival (Fig. 3CII ) or chemotherapy response (Fig. 3DII ). In Fig. 3DIII, it was shown that the exonic mutation has an adverse effect on chemotherapy recipient patients from recovering. In contrast, the exonic mutation has no significant impact on the OS of pediatric Patients with ALL, as it has a p-value of 0.3.

Figure 3: Mutation status and Kaplan-Meier overall survival (OS) curves for pediatric acute lymphoblastic leukemia based on treatment and clinicopathological features. A) The whole cohort of the PAX5 and TP53 gene mutation percentage in pediatric patients with ALL (I), whereas II and III depicted the distribution of wild-type and mutant-type in B-ALL and T-ALL, respectively. B) Overall Survival (OS) curve by Kaplan-Meier method showing the chemotherapy effectiveness as treatment in pediatric Patients with ALL with a significant p-value of 0.011 C) I-III Representing the OS curve by the Kaplan-Meire method based on gender, cancer subtypes, and overall mutational effects on whole cohorts. OS curve between gender (male vs. female) shows statistical insignificance due to a p-value of 0.071 (I). Cancer subtypes (B-ALL and T-ALL) show statistical insignificance in the OS curve with a p-value of 0.72 (II). Overall mutational effects on the exonic region in Patients with ALL also show statistical insignificance with a p-value of 0.3. D) I-III Representing the overall survival (OS) by the Kaplan-Meier method based on gender, cancer subtypes, and overall mutational effects on chemotherapy cohorts. OS curve between gender (male vs. female) shows statistical insignificance due to a p-value of 0.068. Cancer subtypes (B-ALL and T-ALL) also show statistical insignificance in the OS curve with a p-value of 0.98 (II). Overall mutational effects on the exonic region in chemotherapy recipient Patients with ALL show statistical significance with a p-value of 0.0013, which suggests the mutation has an adverse effect on chemotherapy response.

Figure 4: Kaplan-Meier overall survival (OS) curves for pediatric acute lymphoblastic leukemia based on gender, cancer subtypes, and treatment in intronic site mutation. A) For Chr17:7674089, A-C intronic site mutation in the TP53 gene was found significant in cancer types (II) with a p-value of 0.0051, whereas no significance was found on the OS curve based on gender and treatment in this mutation site. B) For Chr17:7674109, G-A intronic site mutation in the TP53 gene was also found significant in cancer types (II) with a p-value of 0.039. In contrast, no significance was observed on the OS curve based on gender (I) and treatment (III) in this mutation site. C) For Chr9: 37020625 A-C intronic site mutation in the PAX5 gene, no significance was observed on the OS curve based on none of the gender (I), cancer types (II), and treatment (III) in this mutation site.

Fig. 4A shows the OS curve for the TP53 gene Chr17:7674089 (A-C) intronic site mutation based on
gender, subtypes, and treatment approach. Mutant T-ALL has a less survival rate than mutant B-ALL (p-value 0.0051). Gender and treatment approach has no significance in OS. The OS curve for the TP53 gene Chr17:7674109 (G-A) intronic site mutation is displayed in Fig. 4B, categorized by gender, subtypes, and treatment strategy. The survival rate for mutant T-ALL is lower than that of mutant B-ALL, with a p-value of 0.039. Chemotherapy has shown a better survival rate than non-chemotherapy despite no statistical significance, with a p-value of 0.063. Fig. 4C displays the OS curve of the intronic site mutation Chr9:37020625 (A-C) in the PAX5 gene located on Chr9. The data is categorized based on gender, subtypes, and treatment strategy. However, no statistical significance was found in either of these OS curves.

4. Discussion:
Pediatric acute lymphoblastic leukemia (ALL) is a type of cancer that affects the white blood cells, specifically the lymphocytes. The development of ALL is a complex process that involves multiple genetic and epigenetic changes. In the ALL-patients cohort, the effect of chemotherapy on patient survival and their relations with mutations in PAX5 and TP53 genes are also investigated through the overall survival using the Kaplan-Meier method.

Among the patients with ALL sequenced in this study, T-ALL comprised 18.33% (11/60), and B-ALL accounted for 81.67% (49/60) of the total cases, which is consistent with the literature review. The male: female ratio was 1.14:1, including 32 males and 28 females, which is almost similar to another study. The median age of the patients was 5 years (range, 0.2-12 years). Several genes have been identified as playing a role in ALL development, including TP53, PAX5, and JAK2. In this study, our attention will be directed toward examining the clinical significance of the emergence of gene mutations and how they affect the growth and progression of ALL.

TP53 gene mutations have been found in certain pediatric ALL in roughly 4-5% of all cases, with a greater prevalence in specific disease subtypes. According to some other studies, TP53 gene abnormalities were found in approximately 6% of patients with pediatric ALL. Understanding the association between ALL treatment results and TP53 gene alterations is necessary. Recent studies have found that TP53 gene mutations have been associated with a poorer response to chemotherapy and a worse prognosis. One study found that patients with ALL who had TP53 gene abnormalities had a lower overall survival rate and event-free survival rate than patients without TP53 gene abnormalities. Another study found that TP53 gene abnormalities were associated with a higher risk of relapse and a lower response to chemotherapy in patients with ALL. Still, the relationship between TP53 gene mutations and chemotherapy outcomes varies depending on the specific type of ALL and other factors associated with ALL. Sanger sequencing revealed that TP53 mutations were present in the exonic region in about 3.33% (2/60) of the total cases, with the remaining 58 patients (96.67%) having wild-type TP53 alleles. Our results were almost similar to a recent study where mutations found in pediatric ALL about 4%. TP53 mutation at codon 167 in the exon 5 mutation site. Moreover, codon 289 in exon 8 is a novel mutation detected on TP53. In TP53, codon 167 was changed from C AG-A AG, resulting in Gln167Lys, and codon 289 C TC-T TC resulting in Leu289Phe substitution.

Our research discovered two new mutations in the intronic region located at Chr17:7674109 and Chr17:7674109. Cytosine (C) was replaced by adenine (A) in Chr17:7674089, whereas guanine (G) was replaced by adenine (A) in Chr17:7674089. 12 (20%) patients were mutated in the intronic site (Chr17:7674089) of the TP53 gene. This intronic site mutation in the TP53 gene was significant between B-ALL and T-ALL with a p-value of 0.0051. T-ALL was a worse prognosis than B-ALL (Fig. 4AII). The study analyzed mutational effects in whole cohorts and patients who received chemotherapy. None of them were statistically significant (Fig. S1F-G).

On the other hand, there were 8% mutated cases among the 60 pediatric Patients with ALL in Chr17:7674109 (G-A) of the TP53 gene. In this intronic site mutation of the TP53 gene, patients diagnosed with T-ALL were found to have a worse prognosis than B-ALL, with a p-value of 0.039 (Fig. 4BII). Chemotherapy vs. non-chemotherapy recipient patients was not found prognosis difference based on current observation.
(p-value 0.063). In addition, no statistical significance was observed in the occurrence of mutations at this site among the whole cohort and those who received chemotherapy. The p-values were 0.42 and 0.99, respectively.

PAX5 gene mutations have been identified in a certain percentage of ALL cases, and they are more common in specific subtypes of the ALL. In one study, PAX5 gene mutations were identified in approximately 5% of ALL cases. Another study found that PAX5 gene mutations were present in approximately 7% of cases of ALL. It is also important to note that the treatment of ALL is complex and typically involves a combination of chemotherapy, radiation therapy, and other therapies, depending on the specific characteristics of the cancer. The role of PAX5 gene mutations in response to chemotherapy in patients with acute lymphoblastic leukemia (ALL) has yet to be fully understood. Studies have suggested that PAX5 gene abnormalities may be associated with a poorer response to chemotherapy and a worse prognosis in some ALL cases. One study found that patients with ALL who had PAX5 gene abnormalities had lower overall survival and event-free survival rates than patients without PAX5 gene abnormalities. The study also found that patients with ALL who had PAX5 gene abnormalities had a higher risk of relapse and a lower response to chemotherapy. In our study cohort, 5% of cases (3/60) carried the PAX5 mutation, and the rest of the 95% (57/60) carried wild type PAX5 gene. The mutation frequency of the PAX5 gene is also similar to a recent study. The PAX5 mutation was observed in the hotspot region's codon 26 of exon 2. Furthermore, codons 84 and 91 in exon 3 were novel mutations detected on PAX5. In the PAX5 gene mutation, codon 26 was changed from GT T-GG T, resulting in Val26Gly substitution. In addition, codons 84 and 91 were altered to GG A - GA A and GC CC - CC resulting in Gly84Glu and Ala91Pro substitution, respectively (Fig. 2B). In a total of 3 mutations, 2 present in B-ALL with a mutation percentage of about 4.08%. On the other hand, one T-ALL patient (9.91%) carried a PAX5 mutation. Differences in PAX5 mutation frequency were observed between B-ALL and T-Patients with ALL in this cohort. The mean age of the PAX5 mutated cases is 3 (range 2-4 years), and all cases occurred in female patients (Table S1).

Upon analyzing the PCR amplicon of the PAX5 gene, a new mutation has been detected in the intronic region located at Chr09:37020625. Adenine (A) was replaced by cytosine (C), and about 65% (39/60) of patients were mutated, and male patients were frequently mutated compared to females. There was no statistical significance in the occurrence of mutations for the whole cohort and chemotherapy recipient patients. The p-values for these groups are 0.99 and 0.46, respectively (Fig. S3E-F). There was also no significance in the mutated sample based on gender, cancer type, and treatment strategy.

In conclusion, the PCR amplicon sanger sequencing results showed mutations in the TP53 and PAX5 genes in pediatric ALL, while the JAK2 gene had no mutation. B-ALL had a higher frequency of exon mutations. All exon mutation events were in female patients. The study also identified 3 novel intronic site mutations, with 2 of them located in the TP53 gene showing worse prognosis in T-Patients with ALL. TP53 exon mutation was associated with poor response to chemotherapy. Further research on TP53 and PAX5 gene mutations in the pediatric ALL population in Bangladesh is necessary to develop personalized treatment plans.

Conflict of Interest:
The authors declare they have no conflicts of interest.

Authors contribution:
Hossain Uddin Shekhar conceived the study. Mohd. Faijanur – Rob Siddiquee, Md. Ismail Hosen and Hossain Uddin Shekhar designed the experiments. Mohd. Faijanur – Rob Siddiquee wrote the manuscripts, analyzed and interpreted the results. Mohd. Faijanur – Rob Siddiquee, Syeda Jarka Jahir, and Fatema Akter performed Dry and wet lab experiments, sample collection, and data collection. Md. Ismail Hosen, Amirul Morshed Khasru, and Hossain Uddin Shekhar performed the English Editing and Proofreading. Amirul Morshed Khasru and Hossain Uddin Shekhar Supervised the experiments and Research. All authors approved the final version of the manuscript.
Acknowledgment:
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References:

Table Legends:

Table 1: Primer sequences of targeted gene (TP53, PAX5, and JAK2) region with their product sizes (bp) and optimum temperature (Tm) from gradient PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Target Region</th>
<th>Primer Sequence (5’ – 3’)*</th>
<th>Product Size (bp)</th>
<th>Optimum Temperature ()</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>Exon 5 (Codon 154, 167, 176)</td>
<td>F: TCACTTGTGC-CCTGACTTTCA R: AATCAGTGGAGGAATACAGAGGCC</td>
<td>304</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Exon 7 (Codon 245, 248)</td>
<td>F: TCACTTGGGC-CTGTGTTATCT R: ACAGGTTAAGAGGTCCCAAGC</td>
<td>332</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Exon 8 (Codon 273)</td>
<td>F: ATGGGACAGGTAG-GACCTGATT R: GCATAAATGCACCCCTTGCT</td>
<td>250</td>
<td>56</td>
</tr>
<tr>
<td>PAX5</td>
<td>Exon 2 (Codon 26, 41)</td>
<td>F: AGCGGT-GCTTCTCCTAT-GTGA R: CATAATTGGACAGCTGCTGGGT</td>
<td>313</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Exon 3 (Codon 80)</td>
<td>F: ACTTTACTG-GTTCCTCATG-GCT R: AGACCAGATCTTCAGGAAAGGC</td>
<td>337</td>
<td>56</td>
</tr>
<tr>
<td>JAK2</td>
<td>Exon 16 (Codon 683)</td>
<td>F: GGGGCTTGAA-CATACTAAATGC R: CAACATGCCCCTTTACACC</td>
<td>281</td>
<td>54</td>
</tr>
</tbody>
</table>

Table 2: Clinicopathological characteristics of pediatric acute lymphoblastic leukemia patients in this study.

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Category</th>
<th>No. of patients N= 60 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Males</td>
<td>32 (53.3%)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>28 (46.7%)</td>
</tr>
<tr>
<td>Immunophenotypes</td>
<td>B ALL</td>
<td>49 (81.7%)</td>
</tr>
<tr>
<td>Clinical Features</td>
<td>Category</td>
<td>No. of patients N= 60 (%)</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>T ALL</td>
<td></td>
<td>11 (18.3%)</td>
</tr>
<tr>
<td>Age (year)</td>
<td>&lt; 1</td>
<td>3 (5.0%)</td>
</tr>
<tr>
<td></td>
<td>1-10</td>
<td>47 (78.3%)</td>
</tr>
<tr>
<td></td>
<td>&gt;10</td>
<td>10 (16.7%)</td>
</tr>
<tr>
<td>Median age, years (Range)</td>
<td>5 (0.2-12)</td>
<td>13 (13.63%)</td>
</tr>
<tr>
<td>ΩΒ⁺ ςουντ ξ10³/μΛ</td>
<td>&lt;4.5</td>
<td>14 (9.1%)</td>
</tr>
<tr>
<td></td>
<td>&gt;11</td>
<td>33 (77.27%)</td>
</tr>
<tr>
<td>Μεαν ΩΒ⁺ ςουντ, ξ10³/μΛ (Ράνγε)</td>
<td>69.33 (0.5-560.71)</td>
<td>55 (91.7%)</td>
</tr>
<tr>
<td>Πλατελετ ςουντ ξ10³/μΛ</td>
<td>&lt;100</td>
<td>5 (8.3%)</td>
</tr>
<tr>
<td></td>
<td>[?]&gt;100</td>
<td>55 (91.7%)</td>
</tr>
<tr>
<td>Μεαν Πλατελετ ςουντ, ξ10³/μΛ (Ράνγε)</td>
<td>43. 08 (3-174)</td>
<td>5 (8.3%)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>&lt;7.0</td>
<td>29 (48.3%)</td>
</tr>
<tr>
<td></td>
<td>[?]&gt;7.0</td>
<td>31 (51.7%)</td>
</tr>
<tr>
<td>Mean Hemoglobin (g/dL)</td>
<td>7.2 (2.2-18)</td>
<td>6 (10.0%)</td>
</tr>
<tr>
<td>Blast cells (%)</td>
<td>&lt;25</td>
<td>6 (10.0%)</td>
</tr>
<tr>
<td></td>
<td>[?]&gt;25</td>
<td>54 (90.0%)</td>
</tr>
<tr>
<td>Mean Blast cell count (%) (Range)</td>
<td>61.5% (7-81)</td>
<td>25 (41.7%)</td>
</tr>
<tr>
<td>Treatment</td>
<td>Chemotherapy</td>
<td>25 (41.7%)</td>
</tr>
<tr>
<td></td>
<td>Non-chemotherapy</td>
<td>35 (58.3%)</td>
</tr>
<tr>
<td>Living status</td>
<td>Death</td>
<td>50 (83.3%)</td>
</tr>
<tr>
<td></td>
<td>Alive</td>
<td>10 (16.7%)</td>
</tr>
</tbody>
</table>

**Table S1:** Mutant patients (exon region) of pediatric ALL with their features.

**Table S2:** Mutant patients (Intron region) of pediatric ALL with their features.

**Figure Legends:**
Figure 1: Exon mutation of the TP53 gene in pediatric acute lymphoblastic leukemia. A) Pie chart showing the percentage of mutation among the samples (I), whereas II depicted the percentage of wild-type and mutant-type TP53 genes in B-ALL. B) Chromatograms showing mutation, the red circle marked the mutation position. (I) Gln167Lys (C AG-A AG) substitution, and (II) Leu289Phe (C TC-T TC) substitution. C) Lollipop chart containing the mutation sites of the TP53 gene in the samples. D) Mutational effects on the overall survival of the whole cohort between wild-type and mutant-type in TP53 mutation were statistically insignificant (p-value 0.8). E) Mutational effects of TP53 mutation on the overall survival of the chemotherapy recipient patients were statistically significant (p-value 0.001) and showed poor prognosis in chemotherapy mutant patients.
Figure 2: Exon mutation of the PAX5 gene in pediatric acute lymphoblastic leukemia. A) Pie chart showing the percentage of mutation among the samples (I), whereas II and III depicted the percentage of wild-type and mutant-type PAX5 genes in B-ALL and T-ALL. B) Chromatograms showing mutation, the red circle marked the mutation position. (I) Val26Gly (GT -GG T) substitution, (II) Gly84Glu (GG A-GA A) substitution, and (III) Ala91Pro (GC C- CC CC) substitution. C) Lollipop chart containing the mutation sites on the PAX5 genes in the samples. D) Kmplot represents the mutational effects on the overall survival of the whole cohort between wild-type and mutant-type in PAX5 mutation with a p-value of 0.14 which was statistically insignificant. E) Kmplot represents the mutational effects on overall survival of chemotherapy recipients between wild-type and mutant-type in PAX5 gene also found statistically insignificant with a p-value of 0.087.
Figure 3: Mutation status and Kaplan-Meier overall survival (OS) curves for pediatric acute lymphoblastic leukemia based on treatment and clinicopathological features. A) The whole cohort of the PAX5 and TP53 gene mutation percentage in pediatric Patients with ALL (I), whereas II and III depicted the distribution of wild-type and mutant-type in B-ALL and T-ALL, respectively. B) Overall Survival (OS) curve by Kaplan-Meier method showing the chemotherapy effectiveness as treatment in pediatric Patients with ALL with a significant p-value of 0.011. C) I-III Representing the OS curve by the Kaplan-Meier method based on gender, cancer subtypes, and overall mutational effects on whole cohorts. OS curve between gender (male vs. female) shows statistical insignificance due to a p-value of 0.071 (I). Cancer subtypes (B-ALL and T-ALL) show statistical insignificance in the OS curve with a p-value of 0.72 (II). Overall mutational effects on the exonic region in Patients with ALL also show statistical insignificance with a p-value of 0.3. D) I-III Representing the overall survival (OS) by the Kaplan-Meier method based on gender, cancer subtypes, and overall mutational effects on chemotherapy cohorts. OS curve between gender (male vs. female) shows statistical insignificance due to a p-value of 0.068, which seems to tend to poor
diagnosis in females. Cancer subtypes (B-ALL and T-ALL) also show statistical insignificance in the OS curve with a p-value of 0.98 (II). Overall mutational effects on the exonic region in chemotherapy recipient patients with ALL show statistical significance with a p-value of 0.0013, which suggests the mutation has an adverse effect on chemotherapy response.

Figure 4: Kaplan-Meier overall survival (OS) curves for pediatric acute lymphoblastic leukemia based on gender, cancer subtypes, and treatment in intronic site mutation. A) For Chr17:7674089, A-C intronic site mutation in the TP53 gene was found significant in cancer types (II) with a p-value of 0.0051, whereas no significance was found on the OS curve based on gender and treatment in this mutation site. B) For Chr17:7674109, G-A intronic site mutation in the TP53 gene was also found significant in cancer types (II) with a p-value of 0.039. In contrast, no significance was observed on the OS curve based on gender (I) and treatment (III) in this mutation site. C) For Chr9: 37020625 A-C intronic site mutation in the PAX5 gene, no significance was observed on the OS curve based on none of the gender (I), cancer types (II), and treatment (III) in this mutation site.

Figure S1: Chr17:7674089 A-C intronic mutation of TP53 gene in pediatric ALL. A) Pie chart showing the percentage of the whole cohort of the Chr17:7674089 A-C intronic TP53 gene mutation percentage in Patients with ALL (I), whereas II and III depicted the percentage of wild-type and mutant-type TP53 gene in B-ALL and T-ALL. B) Chromatograms showing a mutation in Chr17:7674089 (A-C) of TP53 gene; C-E) Donut chart displaying the percentage of patients based on gender, subtypes, and treatment in mutated Patients with ALL. F-G) Kmplot represents the mutational effects on overall survival of the whole cohort (F) and chemotherapy recipient (G) between wild-type and mutant-type in TP53 were statistically insignificant.

Figure S2: Chr17:7674109 G-A intronic mutation of TP53 gene in pediatric ALL. A) Pie chart showing the percentage of the whole cohort of the Chr17:7674109 G-A intronic TP53 gene mutation percentage in Patients with ALL (I), whereas II and III depicted the percentage of wild-type and mutant-type TP53 gene in B-ALL and T-ALL. B) Chromatograms showing a mutation in Chr17:7674109 (G-A) of TP53 gene; C-E) Donut chart displaying the percentage of patients based on gender, subtypes, and treatment in
mutated Patients with ALL. F-G) Knnplot represents the mutational effects on overall survival of the whole cohort between wild-type and mutant-type in TP53 gene statistically insignificant (p-value 0.42) and the mutational effects on overall survival of chemotherapy recipients (G) between wild-type and mutant-type of TP53 gene also statistically insignificant with a p-value of 0.99.

Figure S3: Chr09:37020625 A-C intronic mutation of PAX5 gene in pediatric ALL. A) Pie chart showing the percentage of the whole cohort of the Chr9:37020625 A-C intronic PAX5 gene mutation percentage in Patients with ALL; B-D) Donut chart displaying the percentage of patients based on gender, subtypes, and treatment in mutated Patients with ALL. E) Knnplot represents the mutational effects on overall survival of the whole cohort between wild-type and mutant-type in PAX5 mutation is statistically insignificant with a p-value of 0.99; F) Knnplot represents the mutational effects on overall survival on chemotherapy recipient between wild-type and mutant-type in PAX5 mutation with a p-value of 0.46 which is also statistically insignificant.