BTK mutations selectively regulate BTK expression and upregulate monocyte XBP1 mRNA in XLA patients

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Abstract
Mutations in the Bruton agammaglobulinemia tyrosine kinase (BTK) gene are responsible for X-linked agammaglobulinemia (XLA). Unfolded or misfolded proteins can trigger stress pathways in the endoplasmic reticulum (ER), known as unfolded protein response (UPR). The aim was to clarify the involvement of UPR in XLA pathophysiology. By reverse transcription-quantitative PCR, we evaluated the expression of BTK and 12 UPR-related genes in eight patients. Moreover, we assessed the BTK protein expression and pattern in the patients’ monocytes by flow cytometry and fluorescence immunocytochemistry. We found a reduced BTK expression in patients with stop codon mutations (P < 0.02). However, missense mutations did not affect BTK expression. Flow cytometry showed a reduction of BTK in patients which was corroborated by an absent or nonfunctional protein synthesis revealed by immunocytochemistry. In contrast with the other UPR-related genes, X-box binding protein 1 (XBP1) was markedly upregulated in the patients (P < 0.01), suggesting Toll-like receptor (TLR) activation since BTK directly interacts with TLRs as a negative regulator and XBP1 can be activated in direct response to TLR ligation. Different BTK mutations can be identified by the BTK expression. Inasmuch as UPR-related genes were downregulated or unaltered in patients, we speculate the involvement of the TLRs-XBP1 axis in the XLA pathophysiology. Such data could be the basis for further studies of this novel pathomechanism concerning XLA.

Introduction
X-linked agammaglobulinemia (XLA, OMIM # 300755) is characterized by an end in differentiation of B lymphocytes in bone marrow, leading to profound hypogammaglobulinemia, few or absent peripheral B lymphocytes, and recurrent infections with encapsulated bacteria and enteroviruses [1, 2]. Bruton agammaglobulinemia tyrosine kinase (BTK) is the gene which causes XLA when functionally mutated [3, 4]. It encompasses 37.5 kb containing 19 exons and encodes a multidomain protein composed of five different domains [4, 5].

Mutations have been found in all BTK domains, being spread throughout the gene (http://bioinf.uta.fi/BTKbase) and have been reported in many different countries [6]. Our group has been the first to publish the diagnosis of XLA by
analysis of mutations of the BTK gene in Brazilian patients [7, 8]. Despite the large number of mutations identified, it has not been possible to correlate the genetic defect with the severity of the resulting clinical phenotype [9]. Expression analyses in XLA patients have shown that most of these individuals do not express BTK protein regardless of the mutations they have [10–13]. However, it is not clear how specific mutations may affect the function of BTK and cause XLA.

Unfolded or misfolded proteins can trigger stress pathways in the endoplasmic reticulum (ER), called unfolded protein response (UPR). Stress pathways of the ER have been implicated in inflammatory diseases [14] but not yet studied in XLA patients. There are three ER stress branches of sensors: ERN1 (endoplasmic reticulum to nucleus signaling 1) (also known as IRE1 [inositol-requiring protein 1]), EIF2AK3 (eukaryotic translation initiation factor 2-alpha kinase 3) (also known as PERK [PKR-like-ER kinase]), and ATF6 (activating transcription factor 6). The ERN1-XBP1 (X-box binding protein 1) pathway is the most highly conserved and is critical for ER biogenesis and the secretory capacity of cells [15]. The amount of XBP1 mRNA is critical to the production of the active (spliced) form of (s)XBP1 [16]; the XBP1 mRNA level is kept low in non-stressed cells and its transcription is induced under ER stress [17]. As UPR is constitutively active at a basal level [18–20], regulation through induction or repression of XBP expression allows the maintenance of ER homeostasis [15]. Moreover, XBP1 activity is regulated at multiple levels and may modulate ER homeostasis independently of classic UPR activation [15]. Studies demonstrated that XBP1 plays a role in innate and adaptive immune responses such as in the terminal differentiation of B lymphocytes to plasma cells [21], effector CD8+ T cells [22], in dendritic cell survival [23], immunoglobulin secretion [24], and macrophage responses induced via Toll-like receptors [25].

In this study, we investigated the relation between the mutated BTK and UPR and report for the first time the overexpression of XBP1 in monocytes from XLA patients. As an important multifunctional transcription factor that controls the expression of critical genes associated with the proper functioning of the immune system and the cellular stress response, the involvement of XBP1 on the XLA pathophysiology is conceivable.

Methods

Subjects

X-linked agammaglobulinemia was diagnosed according to the criteria of the World Health Organization scientific group for primary immunodeficiency diseases [26]: low levels of circulating B cells (measured by levels of CD19-positive cells in blood samples), decreased or absent immunoglobulins in serum, and a typical clinical history with recurrent bacterial infection or a positive family history.

Eight male patients with X-linked agammaglobulinemia (median age 17.73; range from 6.36 to 32.02 years old) and eight healthy male volunteers (median age 18.62; range from 6.39 to 32.45 years old) were enrolled for the study. Patients were in a clinically stable situation without fever and not hospitalized. They were under intravenous human immunoglobulin therapy monthly and had no infectious intercurrences.

Monocyte UPR-related gene expression evaluations were carried out only in six patients (median age 14.36; range from 6.36 to 32.02) because two could not provide an adequate amount of cells. For statistical comparisons, the control group also consisted of six age- and gender-matched healthy volunteers.

This study was approved by the Research Ethics Committee of the University of Campinas (UNICAMP), Campinas, São Paulo, Brazil (#759/2005). Clinical characteristics, including levels of immunoglobulins and B cell number, are described in Table 1.

Detection of BTK mutations

Briefly, peripheral blood mononuclear cells (PBMC) were prepared from venous blood using Ficoll-Hypaque separation. Total RNA was extracted from PBMC with TRIzol® Reagent (Life Technologies, Carlsbad, CA) and used for first-strand cDNA synthesis. Bruton agammaglobulinemia tyrosine kinase PCR amplifications involved seven overlapping primers [10]. To confirm the detected mutation, genomic DNA was purified from venous blood with a Gentra Puregene Blood Kit (QIAGEN, Life Technologies) and amplified with primers encompassing the changed region in the BTK gene [27]. Amplicons were sequenced by an ABI 3730 DNA Analyzer (Life Technologies).

Reverse transcription-quantitative PCR (RT-qPCR)

To extract total RNA, 1 mL of TRIzol® Reagent (Life Technologies) was added per 5–10 × 10^6 of PBMC samples, homogenized, and then further processed according to the manufacturer’s instructions. Subsequently, 1 µg of total RNA of each sample was reverse transcribed into cDNA using 200 U of Superscript III Reverse Transcriptase (Life Technologies) and 3 µg of Random Primers (Life Technologies) again according to the manufacturer’s instructions. To assess the expression of ER stress sensors on monocytes, CD14+ monocyte subpopulation was isolated from PBMC using a magnetic cell sorting system in accordance with the manufacturer’s protocol (Miltenyi Biotec, Bergisch
Gladbach, Germany). Total RNA was extracted from monocytes with an illustra RNAspin Mini Isolation kit (GE Healthcare, Buckinghamshire, UK) and similarly reverse transcribed into cDNA.

Complementary DNA samples derived from the investigated genes (Table 2) were detected using an ABI PRISM 7500 Sequence Detection System (Life Technologies) and TaqMan Gene Expression Assays (Life Technologies): 5’-FAM-labeled probes and corresponding primer pairs. Each qPCR was run as triplicates with a cDNA sample of 10 ng in 6.25 μL of TaqMan Gene Expression Master Mix (Life Technologies), 0.625 μL of the respective probe/primer mix, and 0.625 μL of purified and deionized H₂O. Relative gene expression quantification data were generated and analyzed using the 7500 Software version 2.0.5 (Life Technologies). Expression levels of genes were calculated with the 2⁻¹ΔΔCt method using the combination of GAPDH and HPRT1 as the reference gene.

Flow cytometry analysis of BTK expression

BTK protein quantification was calculated by flow cytometry [8, 28]. PBMC were removed from whole blood using Ficoll-Hypaque and later were stained with a phycoerythrin-labeled anti-CD14 (IgG2a; Dako, Japan) monoclonal antibody to separate monocytes. These cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature, permeabilized in 0.1% Triton X-100 for 5 min, incubated with 2 μg/mL anti-BTK (48-2H) or control IgG1 (Dako) monoclonal antibodies for 20 min on ice. Following this, they were washed and incubated again with a 1:1000 dilution of fluorescein isothiocyanate-conjugated goat antimouse IgG1 antibody (SouthernBiotech, Birmingham, AL) for 20 min on ice. The stained cells were analyzed by flow cytometry (Epics XL-MCL flow cytometer, Beckman Coulter, Pasadena, CA).

Immunocytochemistry and confocal fluorescence microscopy

Peripheral blood mononuclear cells were pipetted onto a glass slide previously treated with acetic acid 2 M and 70% ethanol. Adherent cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature. For the process of permeabilization, cells were treated with 0.2% Triton X-100 in PBS for 20 min.

Non-specific binding sites were blocked using 6% goat serum. Cells were incubated overnight at 4°C with monoclonal anti-BTK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), that recognizes the epitope at amino acid positions 459-659 of the human BTK. Further, we used a FITC-conjugated goat anti-mouse IgG1 as the secondary antibody (SouthernBiotech – 1:1000). After extensive

Table 1. Clinical and laboratorial characteristics of X-linked agammaglobulinemia patients.

| Patient  | Age (years) | Age at onset | Age at diagnosis | Family history | IgG levels at diagnosis (mg/dL) | CD19+ (%) | BTK expression | Mutations |
|----------|-------------|--------------|------------------|----------------|-------------------------------|---------|----------------|-----------|
| XLA01    | 20.67       | 4 y          | 6 y              | +              | 298 (750–1,780)              | 90–450  | 26.30%         | 215G > T  |
| XLA02    | 24.11       | 6 y          | 6 y              | +              | 180 (750–1,780)              | 90–450  | 25.60%         | R28L PH   |
| XLA03    | 24.76       | 6 y          | 6 y              | +              | 126 (750–1,115)              | 90–450  | 23.90%         | G653S X55 |
| XLA04    | 9.31        | 2 y          | 2 y              | +              | 81 (610–1,610)               | 40–289  | 3.00%          | 6194T A   |
| XLA05    | 32.02       | 2 y          | 8 y              | +              | 54 (750–1,780)               | 90–450  | 13.30%         | 1634T C   |

UD: undetectable. *Normal expression is >95%.

*Indicates that boy(s) in the same family died at a young age because of infection.
washing with PBS, the coverslips were incubated with Hoechst (Life Technologies) for visualization of the nucleus and mounted on slides using Prolong Gold antifade (Life Technologies). Images were generated in a confocal fluorescence microscope (LSM - 510 Meta, ZEISS, Jena, Germany) with a magnification of 63×.

Statistical analysis
The GraphPad Prism 5 software for Windows (version 5.04) was used for statistical analyses (San Diego, CA; www.graphpad.com). Kruskal–Wallis and Dunn’s Multiple Comparison tests were used to evaluate differences in the BTK expression among three groups: XLA patients with mutations that lead to a premature stop codon, XLA patients with missense mutations, and healthy controls. Regarding the expression of UPR-related genes, Mann–Whitney U test was applied to assess significant differences between XLA patients and controls. Statistical significance was determined as \( P < 0.05 \).

Results
Stop codon or missense mutations were identified in the XLA patients
Table 1 shows the nucleotide change which occurred in each XLA patient and its consequences in the synthesis of the protein.

Four patients had missense mutations, where the substitution of one nucleotide (XLA01 215G>T, XLA02 1970G>A, XLA03 251A>G, and XLA08 1634T>C) leads to an aminoacid replacement in the protein, without the creation of a premature stop codon. However, these missense mutations affect known and conserved residues in BTK, with R28 being one of the most affected. In the PH domain, this residue is essential for binding to phosphatidyl inositol, allowing the protein to reach the plasma membrane [29]. Furthermore, in the PH domain, the substitution of residue Y40 has implications for the conformation of the loop between \( \beta \) strands and the binding site with phosphatidyl inositol [30].

Table 2. List of reference and target genes.

| Approved symbol | Task | Assay ID | Approved name | Synonyms |
|-----------------|------|----------|---------------|----------|
| ATF4            | UPR-related target | Hs00999905_m1 | Activating transcription factor 4 | CREB-2, “tax-responsive enhancer element B67”, TAXREB67 |
| ATF6            | UPR-related target | Hs00984006_m1 | Activating transcription factor 6 | “Activating transcription factor 6 alpha,” AT6F6 |
| BAX             | UPR-related target | Hs00166512_m1 | BCL2-associated X protein | BCL2L4 |
| BCL2            | UPR-related target | Hs00166513_m1 | B-cell CLL/lymphoma 2 | Bcl-2, PPP1R50, “protein phosphatase 1, regulatory subunit 50” |
| BTK             | XLA target | Hs00163761_m1 | Bruton agammaglobulinemia tyrosine kinase | ATAK, PSCTK1, XLA |
| CALR            | UPR-related target | Hs00189032_m1 | Calreticulin | “Autoantigen Ro,” cC1qR, CRT, FLJ26680, RO, “Sicca syndrome antigen A (autoantigen Ro; calreticulin),” SSA |
| DDIT3           | UPR-related target | Hs00999905_m1 | DNA-damage-inducible transcript 3 | “C/EBP zeta,” CHOP, CHOP10, GADD153 |
| EIF2AK3         | UPR-related target | Hs00999905_m1 | Eukaryotic translation initiation factor 2-alpha kinase 3 | PEK, PERK |
| EIF2S1          | UPR-related target | Hs00166512_m1 | Eukaryotic translation initiation factor 2, subunit 1 alpha, 35 kDa | EIF-Zalpha, EIF2A |
| ERN1            | UPR-related target | Hs00166513_m1 | Endoplasmic reticulum to nucleus signaling 1 | “Inositol-requiring enzyme 1,” IRE1, IRE1P |
| GAPDH           | Reference gene | Hs00999905_m1 | Glyceraldehyde-3-phosphate dehydrogenase | |
| HPRT1           | Reference gene | Hs00999909_m1 | Hypoxanthine phosphoribosyltransferase 1 | HGPRT, “Lesch-Nyhan syndrome” |
| HSPA5           | UPR-related target | Hs009999174_m1 | Heat shock 70kDa protein 5 (glucose-regulated protein, 78 kDa) | Bip |
| HSP90B1         | UPR-related target | Hs00427665_g1 | Heat shock protein 90 kDa beta (Grp94), member 1 | GP96, GRP94 |
| IL6             | Interleukin | Hs00958639_m1 | Interleukin 6 | BSF2, HGF, HSF, IL-6, “interferon, beta 2” |
| XBP1            | UPR-related target | Hs00231936_m1 | X-box binding protein 1 | |

Gene names are in accordance with the approved symbol from the HUGO Gene Nomenclature Committee (HGNC) database.
G613 is part of a structural assembly of functionally important residues of the BTK kinase domain [31]. The G613D mutation may prevent the interaction of this domain with other BTK domains or with their substrates. The M501T mutation also affects the kinase domain, suggesting an interference with the enzymatic activity of BTK.

In four patients, XLA04 to XLA07, we identified premature terminations caused by nonsense mutation (718C>T), frameshifts (63104delT and 62854_62855insT), and a splice donor-site defect (IVS5+1G>A).

Different BTK mutations lead to a specific expression profile for BTK

A distinct pattern of BTK expression was observed in patients with a premature stop codon and those with missense mutations (Fig. 1). Specifically, patients with mutations resulting in a premature stop codon exhibit reduced expression of the BTK gene ($P < 0.02$, regardless of the reference gene). However, missense mutations do not affect BTK expression.

Absent or abnormal subcellular localization of BTK protein in the cell cytoplasm

The monocyte BTK expression evaluated by flow cytometry revealed a BTK deficiency (3.0–39.0%) in the seven patients analyzed (Table 1). By immunocytochemistry, we found that mutations in BTK lead to a lack of BTK protein or nonfunctional protein synthesis, evidenced by abnormal subcellular localization of BTK protein in the cell cytoplasm (Fig. 2). The absence of functional BTK is further confirmed by the very low levels of circulating B lymphocytes observed in all patients studied (CD19+ percentage; Table 1).

Increased expression of XBP1 in monocytes from XLA patients

To elucidate the molecular features of UPR on XLA patients, RT-qPCR analysis was performed on CD14+ cells isolated from six patients with XLA and six healthy volunteers. Among the 12 evaluated genes, the expression of XBP1 was significantly higher ($P = 0.0022$) in XLA patients than in healthy volunteers (Fig. 3). On the other hand, HSP90B1 expression was lower in XLA patients ($P = 0.026$).

Additionally, we also quantified IL6 expression. It is known that sXBP1 induces IL6 secretion. We found an IL6 upregulation ($P < 0.02$, GAPDH+HPRT1 as the reference gene) when patients ($n = 7$) were compared to controls ($n = 8$). The patient labeled as XLA05 was excluded from this RT-qPCR experiment because of the total consumption of his RNA template.

Discussion

The definitive diagnosis of XLA is performed by sequencing techniques since in 90–95% of cases, it is possible to identify gene mutations in BTK [32].

According to our results, mutations in BTK that create a premature stop codon (PTC) can be differentiated due to the reduced level of BTK mRNA expression (Fig. 1). These findings are explained by nonsense codons causing a reduction in the mRNA level [33]. The nonsense-mediated mRNA decay (NMD) is a surveillance pathway whose main function is to reduce errors in gene expression by eliminating mRNA transcripts that contain PTCs. According to the literature, it is estimated that 30% of known mutations associated with disease are because of mRNAs containing PTCs [34].

At the protein level, flow cytometry assays revealed a downregulation of BTK for all patients (Table 1). We also
found that mutations in BTK lead to a lack of BTK protein or a nonfunctional protein synthesis, evidenced by abnormal subcellular localization of BTK protein in the cell cytoplasm (Fig. 2). In its active state, BTK is found close to the inner surface of the plasma membrane. In fact, BTK phosphorylation is linked to membrane localization [35, 36].

Proteins which do not have the required conformation are targets of the cellular mechanism which is designated endoplasmic-reticulum-associated protein degradation (ERAD). This mechanism retrotranslocates misfolded proteins from the ER to the cytosol in order to be degraded via the ubiquitin-proteasome (a protein-degrading complex) [37]. Moreover, the increase of unfolded proteins in the ER creates an imbalance between the demand and capacity of the organelle, which is known as ER stress. This triggers a network of signaling events collectively called UPR.

Since ER stress pathways have been implicated in inflammatory diseases [14] but not yet studied in XLA patients, we evaluated by RT-qPCR, the monocyte expression of 12 genes involved in UPR in XLA patients. These genes encompass the three major ER stress branches: ERN1, EIF2AK3, and ATF6, which mediate changes in gene expression that characterize the UPR process [38]. In general, we found a hyporegulation scenario for their expressions but XBP1 had markedly upregulation (Fig. 3). Accordingly, this significant monocyte XBP1 mRNA overexpression immediately ex vivo could suggest that BTK deficiency may produce ER stress. On the other hand, XBP1 can be expressed independently of the ER-stress response.

Toll-like receptor activation causes XBP1 splicing to increase, XBP1 protein to rise, and pro-inflammatory cytokine transcripts to be generated without initiating ER stress responses. As BTK directly interacts with TLRs and may inhibit TLR-induced cytokine production, as suggested by Marron et al. [39], this might be one important explanation for our observations (Fig. 3), which would indicate that the increase in XBP1 transcripts in XLA patients is independent of classical ER-stress response. Moreover, downstream ER-stress target gene expressions, such as HSPA5 and DDIT3, were not significantly altered in our samples, which is evidence that the observed XBP1 overexpression may have occurred in the absence of a classic ER-stress response.

There are no reports in the literature linking BTK and XBP1. Our results prospectively uncover a new mechanism for XBP1 and BTK in the innate immune system. Martinon...
et al. [25] demonstrated a new function of the IRE1-XBP1 pathway in macrophages that is ER-stress independent. They reported that TLR2 and TLR4 specifically activated XBP1 which amplifies TLR signaling by enhancing cytokine production. Also, the IL6 overexpression found in our XLA patients (Fig. 4) corroborates the suggestion of BTK repressing TLR-induced cytokine production [39].

Iwakoshi et al. [40] demonstrated that sXBP1 induces IL6 secretion, suggesting that, in addition to its important role in UPR, sXBP1 regulates the expression of this cytokine that is essential for plasma cell differentiation and myeloma cell growth. The authors raised an interesting hypothesis in which XBP1 has unidentified functions and target genes that are unrelated to UPR, reinforced by the fact that its role in driving the transcription of at least a subset of ER chaperone genes is redundant [40]. Interleukin 6 might be one such target. Our results also support their hypothesis of the existence of a positive feedback loop between IL6 and XBP1 (that would ensure high quantities of both proteins during plasma cell differentiation). Since plasma cell differentiation is defective in XLA patients, the overexpression of both these genes could represent a physiological attempt to correct this failure. In addition, as IL6 is a potent pro-inflammatory cytokine and XBP1 a multifunctional transcription factor, their involvement in XLA physiopathology is conceivable and further studies should be undertaken.

Before adequate immune globulin treatment, several inflammatory conditions were described as being associated with XLA. Recent data from 128 unique XLA patient responses indicated that a considerable number of XLA patients have symptoms that are consistent with a diagnosis of arthritis, inflammatory bowel disease, or other inflammatory conditions [41]. The suggested positive feedback loop between IL6 and XBP1 should be further investigated in these patients.
In response to LPS, PBMC from XLA patients produced significantly higher amounts of pro-inflammatory cytokines and IL10 compared to controls, and this production was influenced neither by the severity of the mutation nor the affected domain [42]. The authors of this study also demonstrated a predominantly inflammatory response in XLA patients after LPS stimulation and suggested a deregulation of TLR signaling in the absence of BTK. Environmental factors were suggested to be involved in this response [42].

Schmidt et al. [43] demonstrated that Btk is activated by Tlr4 in primary macrophages and is required for normal Tlr-induced Il10 production in multiple macrophage populations. The authors suggest that the decreased Il10 production may be responsible for increased Il6. BTK also plays a critical role in initiating TLR3 signaling. In the absence of BTK, TLR3-induced phosphoinositide 3-kinase (PI3K), AKT and MAPK signaling, activation of NFκB as well as interferon regulatory factor 3 (IRF3), and AP-1 transcription factors were all defective. It was demonstrated that BTK directly phosphorylates TLR3 and, in particular, the critical Tyr759 residue. Loss of BTK also compromises the formation of the downstream TRIF/ receptor-interacting protein 1 (RIP1)/TBK1 complex [44]. Since some XLA patients develop enterovirus encephalitis [45], the participation of a defective TLR3 signaling should be considered as a potential molecular pathomecanism in this condition.

Recently, Savic et al. [46] reported the first link between TLR-dependent XBP1 activation and human inflammatory disease. They showed that TLR-dependent XBP1 activation is operative in the synovial fibroblasts (SF) of active rheumatoid arthritis (RA) patients. They found that the active (spliced) form of (s)XBP1 was significantly upregulated in the active RA group compared to healthy controls and patients in remission. Interestingly and paradoxically, the expression of nine other ER stress response genes was reduced in active RA compared to patients in remission, suggestive of a defective TLR3 signaling should be considered as a potential molecular pathomechanism in this condition.

Up to now, the literature has implied that BTK is critical for B cell development and differentiation, but is also involved in the regulation of other cell types, such as monocytes/macrophages [49], neutrophils [50], and NK cells [51]. BTK has also been implicated in TLR signaling and interacts with TLR4, 6, 8, and 9 and also with MYD88, toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP) (also known as MyD88-adaptor-like protein [Mal]) and interleukin-1 receptor-associated kinase 1 (IRAK1) [52], indicating unexplored roles for this protein in innate and adaptive immunity.

We demonstrated that BTK mRNA is markedly downregulated in XLA patients with stop codon mutations and that missense mutations do not affect BTK expression. Moreover, this is the first study relating XBP1 with BTK mutations and XLA. Monocytes from XLA patients overexpress XBP1, a critical transcriptional factor for ER stress and plasma cell differentiation. Our data suggest that defective BTK might affect XBP1 expression in monocytes. As a multifunctional transcriptional factor, our finding on XBP1 upregulation in XLA patients opens several possible avenues of research that will help us to understand the complex pathophysiology in XLA.

Conclusions

Acknowledgments

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Author Contributions

VDR, MAT, LD’S-L, and MMSV designed the study. VDR, MAT, BMA and MMSV collected patients’ clinical data. VDR identified BTK mutations. VDR did the immunocytochemistry assays, total RNA extractions, and RT-PCRs. MAT carried out qPCRs, analyzed all RT-qPCR data, prepared the figures and tables, and performed statistical analyses. VDR, MAT, and MMSV wrote the manuscript. MMSV conceived the study and supervised all aspects of its development. All authors read and approved the final manuscript.

Conflict of Interest

All authors declare that they have no competing interest.

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