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Bruton tyrosine kinase is commonly overexpressed in mantle cell lymphoma and its attenuation by Ibrutinib induces apoptosis

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ABSTRACT

Mantle cell lymphoma (MCL) is an aggressive B-cell malignancy that characteristically shows overexpression of cyclin-D1 due to an alteration in the t(11;14)(q13;q32) chromosomal region. Although there are some promising treatment modalities, great majority of patients with this disease remain incurable. The B-cell antigen receptor (BCR) signaling plays a crucial role in B-cell biology and lymphomagenesis. Bruton tyrosine kinase (BTK) has been identified as a key component of the BCR signaling pathway. Evidence suggests that the blockade of BTK activity by potent pharmacologic inhibitors attenuates BCR signaling and induces cell death. Notably, the expression levels and the role of BTK in MCL survival are still elusive. Here, we demonstrated a moderate to strong BTK expression in all MCL cases ($n = 19$) compared to benign lymphoid tissues. Treatment of MCL cell lines (Mino or Jeko-1) with a potent BTK pharmacologic inhibitor, Ibrutinib, decreased phospho-BTK-Tyr^{223} expression. Consistent with this observation, Ibrutinib inhibited the viability of both Mino and JeKo-1 cells in concentration- and time-dependent manners. Ibrutinib also induced a concentration-dependent apoptosis in both cell lines. Consistently, Ibrutinib treatment decreased the levels of anti-apoptotic Bcl-2, Bcl-xL, and Mcl-1 protein. These findings suggest that BTK signaling plays a critical role in MCL cell survival, and the targeting of BTK could represent a promising therapeutic modality for aggressive lymphoma

1. Introduction

Mantle cell lymphoma (MCL) is characterized by the translocation t(11;14)(q13;q32), which results in the aberrant expression of the cell cycle protein, cyclin-D1 [1–3]. Alteration in DNA damage response genes and activation of cell survival pathways are also implicated in MCL development [4]. MCL accounts for approximately 2–10% of the non-Hodgkin lymphoma cases, with an annual incidence of 0.51–0.55 per 100,000 people [5]. A great majority of MCL patients have stage III/IV disease and present with generalized non-bulky lymphadenopathy, blood and bone marrow involvement, splenomegaly, and extranodal involvement [1,6]. Great majority of patients exhibit an aggressive disease with a median survival 3–5 years [5,7]. These observations strongly suggest the need to develop effective, yet more selective, therapeutic strategies to cure MCL [3,8].

The B-cell antigen receptor (BCR) signaling is crucial to cell survival during B-cell development and regulates multiple biological processes, including cell proliferation, differentiation, apoptosis, and migration [9–11]. In addition, BCR signaling is implicated in the pathogenesis of B-cell malignancies, including MCL [9,12]. Bruton's tyrosine kinase (BTK) is a cytoplasmic protein and has been identified as a key component of the BCR signaling pathway [13]. Signals mediated by BTK were demonstrated to activate cell survival pathways such as transcription factor nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) [9,14]. In addition, the loss-of-function of BTK in humans was linked to X-linked agammaglobulinemia (XLA) syndrome [13]. XLA is an inherited disorder that is characterized by a severe decrease in immunoglobulin production and virtual absence of mature B-cells [14,15]. Since XLA is largely restricted to B-cell lymphocytes, BTK is considered an attractive target for the selective inhibition of B-cell growth [11].

Ibrutinib (PCI-32765) is an orally bioavailable irreversible, potent and highly selective small molecule inhibitor of BTK [15,16]. The results of phase II clinical trail have suggested that Ibrutinib is

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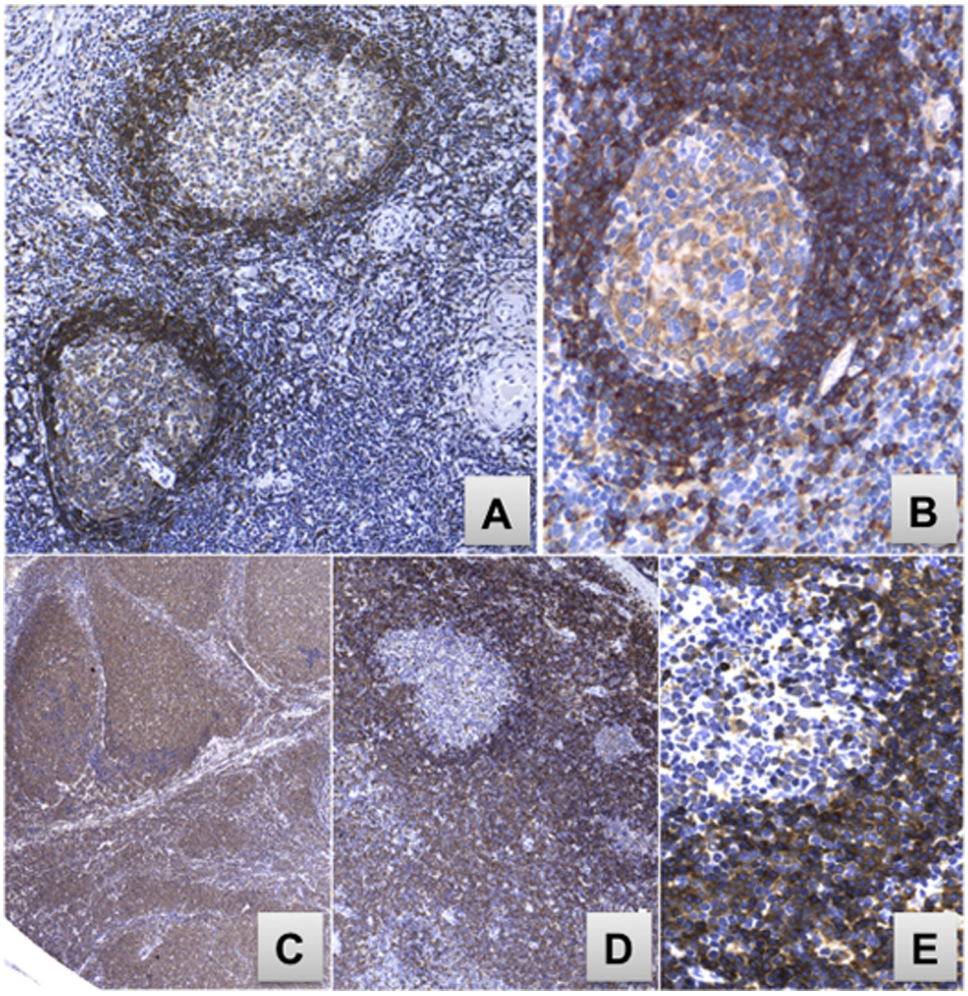

Fig. 1. Immunohistochemical staining of BTK protein in benign lymphoid tissue and mantle cell lymphoma. (A) and (B) BTK expression in benign lymphoid tissue of tonsil with follicular hyperplasia is shown (magnifications A: 100× and B: 400×). Stronger expression of BTK is noticeable in the benign mantle zone cells while a weaker expression noted in the germinal center cells. (C)–(E) Two cases of mantle cell lymphomas are shown. (C) Mantle cell lymphoma primarily with a nodular pattern is illustrating a diffuse expression of BTK by the mantle cell lymphoma cells (magnification: 100×). (D) and (E) Mantle cell lymphoma cells surrounding a residual benign germinal center reveals a strong diffuse cytoplasmic and membranous staining of BTK (D: 100× and E:400×). Micrographs are the representation of multiple images.

likely very effective and well tolerated in relapsed and refractory MCL and the efficacy of this drug is being evaluated in Phase 3 trials [7,10]. Despite these findings, however, the expression of BTK in normal and neoplastic mantle cells as well as the effects of BTK in MCL cell survival has not been systematically assessed.

In the present study, we demonstrated that BTK is commonly overexpressed in MCL compared to benign lymph nodes, and the inhibition of BTK by Ibrutinib attenuated MCL cell growth and survival by a mechanism that involved in the inhibition of anti-apoptotic proteins. These findings suggest that BTK contributes to MCL survival and provide a rationale for the development of novel therapeutic approaches that include BTK inhibitory agents.

2. Materials and methods

2.1. Cell lines and reagents

Mino and JeKo-1 cell lines were grown in RPMI 1640 as described previously [17]. Ibrutinib was obtained from Selleckchem (Houston, TX, USA). Bcl-2 (C-2) PE, Bcl-xL (H-5) FITC, Mcl-1 (H-260), and goat anti-rabbit IgG-PE antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Total and phospho-Tyr^{223}BTK antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Propidium iodide (PI) was purchased from Sigma-Aldrich and 7-amino-actinomycin (7-AAD Viability Dye) from Beckman Coulter (Immunotech, Inc.).

2.2. Immunohistochemistry

Archival formalin-fixed, paraffin-embedded tissue blocks of B-cell lymphoma and benign lymphoid tissue were subjected to immunohistochemistry with BTK on 19 MCL cases along with 10 cases of benign lymphoid tissues. Immunostaining was performed with an automated immunostainer (Leica microsystem). The staining protocol used a rabbit monoclonal antibody against BTK as described by the manufacturer (Cell Signaling Technology). The sections were microscopically evaluated for the intensity of reactivity. The results were recorded in semi quantitative fashion categorized as negative (0), weakly positive (1) or strongly positive (2) cytoplasmic expression in tumor cells based on intensity of the immunostaining.

2.3. Flow cytometry

For the analysis of phospho-BTK-Tyr^{223}, Bcl-2, Bcl-xL, and Mcl-1, cells were processed using intracellular staining kit (Fix & Perm kit, Invitrogen). Cells were labeled with phospho-BTK-Tyr^{223} antibody (1:10 dilution) or with Mcl-1 (1:2 dilutions) for 20 min and washed with PBS, incubated with phycoerythrin-conjugated secondary antibody (goat anti-rabbit IgG-PE) for 30 min at 4 °C, and then washed with PBS before the analysis. Processed-cells were directly stained with Bcl-2-PE or Bcl-xL-FITC conjugated antibody and washed with PBS to remove unbound antibody. Analyses of phospho-BTK-Tyr^{223} at 24 h post treatment and of Bcl-2-PE, Bcl-xL-FITC, or Mcl-1 at 72 h post treatment were performed using Cytomic's FC500 Flow Cytometer (Beckman Coulter).

2.4. Cell viability assay

The viability of Mino and JeKo-1 cells was determined by MTS tetrazolium compound (PromegaCellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay;

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Promega, Madison, WI, USA). Cytotoxicity was assayed by the determination of reduced tetrazolium (formazan) created by metabolically active cells as detected at 490 nm absorbance using a microplate reader (BMG Labtech, Cary, NC, USA).

2.5. Detection of apoptosis

Flow cytometric analysis was performed after staining of the cells with PI and 7-AAD. For PI staining, 0.5×10^6 cells/ml were centrifuged at 1500 rpm for 5 min, supernatant was removed, and cell pellet was gently suspended with 1 ml Krishan buffer and then incubated at 4 °C in the dark for 60 min. Apoptosis was determined by Cytomic's FC500 Flow Cytometer (Beckman Coulter) and the data were analyzed by WinCycleMulticycleSoftware (De Novo Software). [18]. For the 7-AAD staining, 0.5×10^6 cells were washed with PBS and centrifuged at 1500 rpm for 5 min. Supernatant was removed and the cell pellet was suspended in 100 μL PBS. 10 μL 7-AAD was added to each tube and incubated 20 min at room temperature in the dark. 7-AAD labeled-cells were analyzed by Cytomic's FC500 Flow Cytometry (Beckman Coulter) and FCS Express Flow Cytometry Data Analysis software (De Novo Software). For morphologic analysis of apoptosis by light microscopy, cells were incubated with Mock or with 1, 10 or 20 μM Ibrutinib for 72 h. Control or drug treated-cells were cytospun on glass slides, stained with Wright-Giemsa, and then examined by light microscopy. Morphological changes associated with apoptosis included cellular shrinkage, nuclear condensation, nuclear fragmentation, and formation of apoptotic bodies.

2.6. Statistics

Data are represented as mean ±SEM. The statistical significance between control and test groups was assessed by Student *t*-test. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. BTK expression in MCL

Immunohistochemical staining of BTK was assessed in tissues of MCL patients ($n = 19$) and benign lymphoid tissue of reactive lymph nodes or tonsillary tissue showing reactive lymphoid hyperplasia ($n = 10$). MCL cases were previously diagnosed based on morphological, immunohistochemical and FISH studies that included the typical B-cells co-expressing CD5 and cyclin D1 with t(11;14) translocation in the lymphoma cells. Two of the 19 cases analyzed was subclassified and blastic type of MCL. BTK expression in immunohistochemically stained tissues was evaluated by using light microscopy. The results show that BTK is strongly expressed by the lymphocytes residing within the mantle zone layers of reactive lymphoid tissues (Fig. 1A and B) whereas a weaker expression BTK is noted in the germinal center cells. In addition, scattered individual cells are also noted in the interfollicular areas. Moderate to strong BTK reactivity is observed in all mantle cell lymphomas (19/19) that showed strong diffuse cytoplasmic and membranous staining in the MCL cells (Fig. 1C–E).

3.2. Ibrutinib attenuates phospho-BTK expression in MCL cells

The autophosphorylation of BTK at Tyr^{223} residue was suggested to play a crucial role in BTK activation and signaling. To determine whether Ibrutinib inhibits phospho-BTK-Tyr^{223} in MCL, Mino or JeKo-1 cells were treated with 1 or 10 μM Ibrutinib and assessed along with control cells without treatment. At 24 h post treatment, the levels of phospho-BTK-Tyr^{223} were assessed by flow cytometry. The results demonstrated that Ibrutinib treatment downregulated phospho-BTK-Tyr^{223} expression in Mino (Fig. 2) and JeKo-1 (not shown) cells.

3.3. Ibrutinib decreases MCL cell viability

To assess whether the inhibition of BTK activity by Ibrutinib decreases cell viability in MCL, Mino and JeKo-1 cells were treated with varying concentrations of Ibrutinib (0, 0.0001, 0.001, 0.01, 0.1,1, 10, 25, 50, or 100 μM). Under these conditions, cell viability was determined by an MTS assay. As shown in Fig. 3A, Ibrutinib

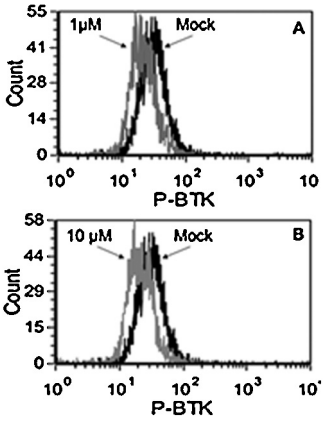

Fig. 2. Analysis of the phospho-BTK expression in Mino MCL cells. Mino cells were treated with 1 μM (A) and 10 μM (B) Ibrutinib or with mock control for 24 h, stained with phospho-BTK antibody, and analyzed by flow cytometry. The data (±SD) are the representation of triplicates.

attenuated the viability of Mino and JeKo-1 cells in a concentration-dependent manner at 72 h post treatment. There was a significant decline ($p < 0.05$) in Mino cell viability, even at the lowest concentration (0.0001 μM) of Ibrutinib. However, a similar level of growth inhibition in JeKo-1 cells was achieved by 1 μM Ibritunib. The IC50 was approximately 1–10 μM for Mino and 10–25 μM for JeKo-1

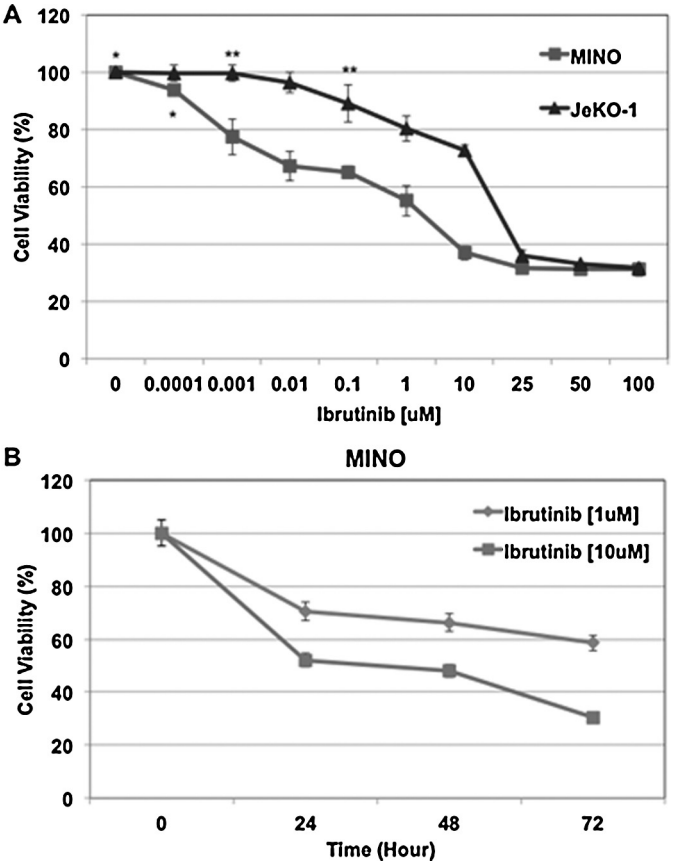

Fig. 3. Effects of Ibrutinib on cell growth. (A) Mino and JeKo-1 cells were treated with increasing concentrations of Ibrutinib or mock control. At 72 h treatment, cell viability was assessed by an MTS assay at 490 nm. (B) Mino cells were treated with two concentrations of Ibrutinib (1 or 10 μM). Both cells were grown in 96-well tissue culture plate. Cell viability was determined by MTS assay at 24, 48, or 72 h post treatments at absorbance 490 nm. Cell viability was presented as the percentage of mock control. The data (±SD) are the representation of independent experiments repeated two times with four data points each; *, **$p < 0.05$.

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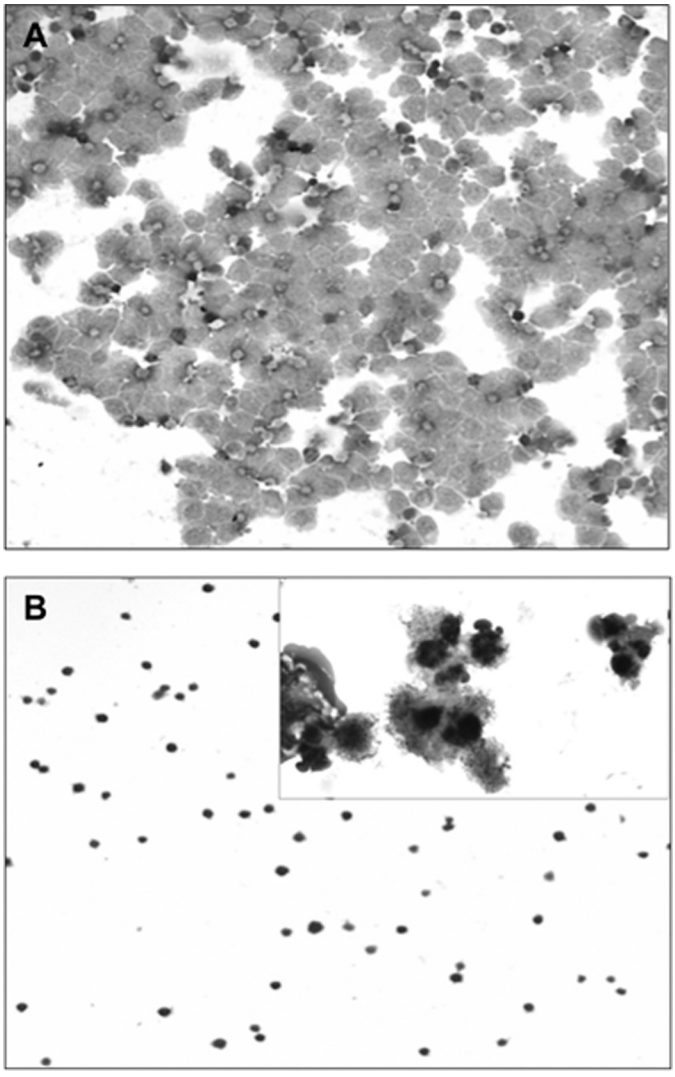

Fig. 4. Morphologic analysis of Ibrutinib-induced cell apoptosis. JeKo-1 cells cultured in 6-well plate were treated with mock control (A) or with 10 μM Ibrutinib (B) for 72 h, and control and drug treated cells were morphologically examined after cytospin preparation. Ibrutinib treated cells show marked cell nuclear shrinkage, fragmentation and budding. Inset shows enlarged cells with fragmentation and apoptotic particles. Micrographs are the representation of multiple experiments.

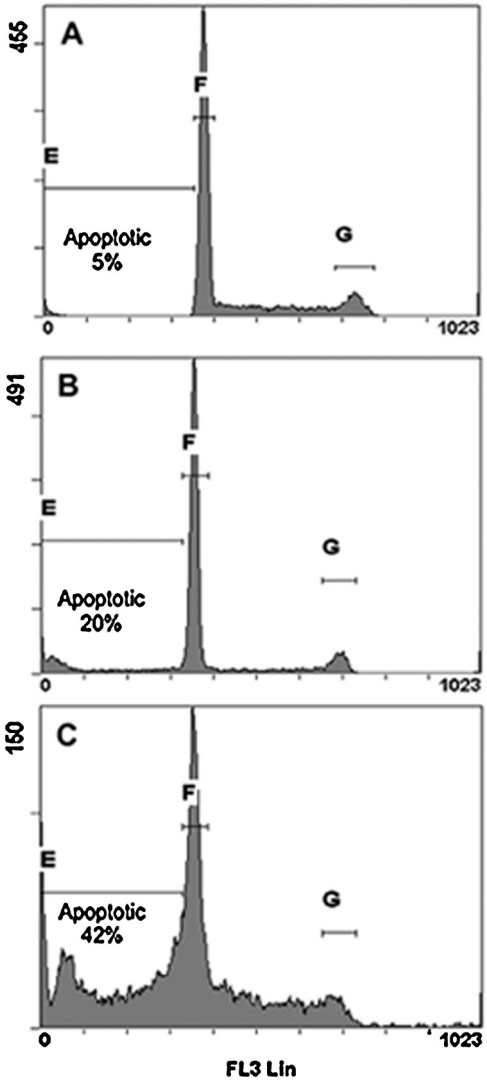

Fig. 5. Analysis of Ibrutinib-induced cell apoptosis by PI staining. Mino cells grown in 6-well plate were treated with mock control (A) or with 10 μM (B) and 20 μM (C) Ibrutinib for 72 hours and stained with PI. The PI-stained cells were analyzed by flow cytometry. Cells treated with 10 or 20 μM Ibrutinib shows statistically significant increase in number of cells in sub-G1/G0 phase compatible with apoptosis compared to mock control. Data are representation of multiple experiments.

cells, as estimated from a sigmoidal concentration curve with variable slope. In addition, the treatment of Mino cells with 1 and 10 μM of Ibritunib for 24, 48, and 72 h showed that Ibritunib significantly reduced the cell viability in a time-dependent manner (Fig. 3B). The inhibition of cell viability was observed as early as 24 h and continued to increase up to 72 h.

3.4. Ibrutinib induces apoptosis

To determine if the inhibition of cell growth by Ibrutinib involves cellular apoptotic pathways, Mino and Jeko-1 cells were treated with mock (control), 10 or 20 μM Ibrutinib for 72 h and cells then were stained with PI or 7-AAD. Under these conditions, apoptosis was determined by morphologic assessment and by flow cytometric analysis. Ibrutinib treatment induced noticeable morphologic changes in Mino (Fig. 4) and Jeko-1 (not shown) cells with apoptosis characteristics: nuclear shrinkage, chromatin condensation and cellular disintegration. To further confirm that these changes were indeed due to occurrence of cell death, PI/7-AAD-stained Mino or JeKo-1 cells were subsequently analyzed by flow cytometry that showed marked apoptosis by Ibritunib treatment (Figs. 5 and 6).

3.5. Ibrutinib downregulates expression of Bcl-2 family proteins

Studies suggested that the levels of Bcl-2 family proteins are altered in response to cell death inducer [19]. In this regard, we examined the levels of anti-apoptotic (Bcl-2, Bcl-xL, and Mcl-1) proteins in Mino or JeKo-1 cells by flow cytometry after treatment of cells with 1 or 10 μM or without Ibrutinib. Compared to the control samples, Ibrutinib treatment significantly decreased the expression of Bcl-2, Bcl-xL or Mcl-1 protein in Mino cells (Fig. 7). However, Ibrutinib treatment had a modest effect on Bcl-2, Bcl-xL, and Mcl-1 protein levels in JeKo-1 cells under the same experimental condition relative to non-drug treated control (not shown). These observations indicate that Mino and JeKo-1 cells responded differentially to the Ibrutinib mediation of cell death induction.

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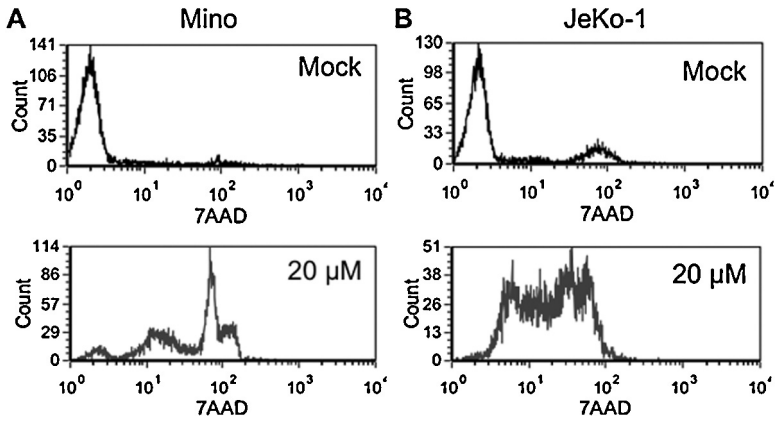

Fig. 6. Analysis of Ibrutinib induced cell apoptosis by 7-AAD. Mino (A) and JeKo-1 (B) cells plated in 6-well were treated with 20 μM Ibrutinib for 72 h. Apoptotic cell population was determined by flow cytometric analysis of 7-AAD stained-cells. A significant increase in percentage of 7-AAD stained positive cells was seen with treatment compatible with apoptosis. The upper histogram in each panel shows untreated control cells, whereas the lower histogram in each panel shows Ibrutinib treatment. The data are the representation of triplicates.

4. Discussion

One of the most important mechanisms contributing to the development of MCL is the overexpression of cyclin-D1 due to t(11;14)(q13;q32) chromosomal translocation that juxtaposes *CCND1* proto-oncogene that is located at chromosome 11q13 next to the immunoglobulin heavy chain gene (*IGH*) region in chromosome 14q32 [20]. It is worth mentioning, however, that the overexpression of cyclin-D1 in lymphoid tissues fails to develop overt lymphoma, but mediates lymphoid hyperplasia. In support of this notion, a minority MCL patients fail to show the deregulation of cyclin-D1 gene, but aberration of a transcription factor, SOX11, that contains high mobility proteins with an ability to promote survival in MCL [21]. This indicates that presence of alternate signaling for cellular transformation and maintenance of survival.

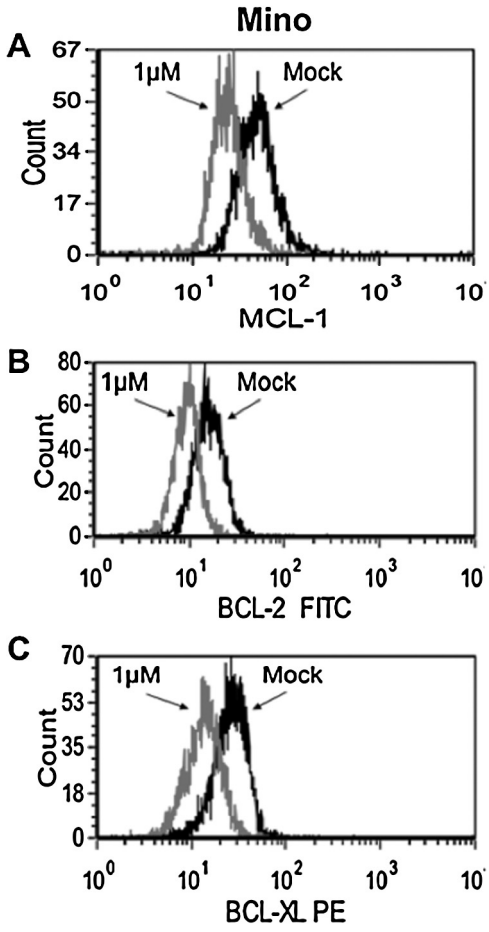

Fig. 7. Effects of Ibrutinib on the expression of anti-apoptotic proteins. Mino cells grown in 6-well plate were treated with mock control or 1 μM Ibrutinib for 72 h, and the levels of Mcl-1 (A), Bcl-2 (B), or Bcl-xL (C) expression were determined by flow cytometry in cells that were labeled with fluorescent-conjugated protein-specific antibody. Ibrutinib treatment reduced the expression of these proteins compared to control. Data are representation of multiple experiments.

In the current study, we showed that BTK-mediated signaling plays an important role in MCL cell growth and survival. BTK protein is commonly expressed by MCL cells. Furthermore, a potent pharmacologic inhibitor of BTK, Ibritunib, inhibits phospho-BTK-Tyr^{223} in Mino and JeKo-1 MCL cells. Ibrutinib treatment also inhibited the growth and viability of MCL cells *ex vivo* and induced apoptosis in a mechanism that involves deduction in the expression of the Bcl-2 family of anti-apoptotic proteins. These findings demonstrate that BTK signaling is a vital for MCL cell survival and may provide a rational for clinical utilizations of BTK.

The BCR signaling regulates cell proliferation, differentiation, and apoptosis of B-lymphocytes [11] and plays a crucial role in the pathogenesis of B-cell malignancies [22]. Investigations of B-cell receptor utilization in the setting of MCL revealed that subpopulation of MCL patients carry IGHV hypermutation and suggested antigen-driven select clonogenic growth of neoplastic MCL cells based on sequence analysis of the complementary-determining region-3 (CDR3) of immunoglobin heavy chain gene [23]. This finding suggests that a subset of MCL developmentally related to clonogenic proliferation of lymphoid cells based on antigen driven selection that is similar to subgroup of chronic lymphocytic leukemia (CLL) patients with select usage of the immunoglobin heavy chain gene. The results from *in vitro* studies and early clinical trials have indicated that the disruption of BTK activity by a potent pharmacologic inhibitor, Ibritunib, has been shown to be a promising therapeutic modality for CLL, [11,14,24,25]. In addition, a recent clinical trial enrolled many patients with relapsed or refractory B-cell lymphoma and CLL and treated with escalating doses of Ibrutinib that lead to marked clinical improvements in many of the cohorts including refractory CLL, follicular lymphoma, diffuse large B-cell lymphoma and MCL [11]. Overall, administration of BTK inhibitor successfully demonstrated objective clinical responses in lymphoma patients, including MCL patients.

BTK is an essential component of the BCR signaling complex and a key mediator of the BRC signaling in B-cell activation and

malignancy [26]. BTK is a phospho-protein and activated by upstream signaling pathway that is linked to B-cell receptor activation. Src family-protein kinase Blk, Lyn, Fyn, or Syk, was demonstrated to phosphorylate BTK, which leads to activation of BTK [27]. Besides, autophosphorylation at Tyr^{223} residue could cause BTK activation [28]. BTK can be activated by BTK promotes the activation of phospholipase C-gamma 2 (PLC-γ2)-protein kinase C (PKC) pathway activation and calcium influx *via* diacylglycerol (DAG) and inositol triphosphate (IP3), respectively [25]. In addition, BTK was demonstrated to mediate the activation of NF-κB and MAP kinase pathway signaling in CLL [14]. This study demonstrated that the inhibition of BTK in CLL contributed to caspase activated apoptosis concurrent with inhibition of number of very critical intracellular proteins with tyrosine kinase activity including Akt and MAP kinases. Given the importance of BTK in B-cell activation and B-cell malignancy, Ibrutinib was developed as a potent BTK inhibitor [29]. The inhibition of BTK by Ibrutinib was shown to prevent B-cell activation in autoimmune disease, leading to a partial or near complete suppression of the clinical symptoms in model organism [16]. This study also showed that Ibrutinib attenuated the growth of lymphoma in canine models. Here, we demonstrated that Ibrutinib significantly attenuated BTK activation as shown by the inhibition of phospho-BTK-Tyr^{223} and that was associated with a significant decline in MCL cell viability *ex vivo*. These findings suggest that the inhibition of BTK activity may display a potent anti-tumor activity.

Evidence based on RNAi studies showed that the attenuation of BTK expression resulted in cell death, possibly due to the disruptions of BCR-initiated and NF-κB-mediated survival signals [10]. In the current study, we showed that Ibrutinib prevented the survival of Mino and Jeko-1 cells in a dose-dependent manner. We noted that Mino cells were more sensitive to the Ibritunib-induced inhibition of cell survival than JeKo-1 cells, given that 1 nM Ibrutinib significantly inhibited Mino cell survival, whereas a similar level of inhibition of JeKo-1 was achieved by 1 μM Ibrutinib. A recently published study by Dashmahapatra et al. [30] showed that Ibrutinib alone has modestly affected the survival of another MCL cell line. Our observations are in agreement with the published study indicating that context dependent events may determine anti-growth activity of Ibritunib in MCL cells.

Furthermore, during the review of our manuscript a phase-2 clinical trial that was designed to investigate the clinical effects of Ibritunib in 111 MCL patients as a single agent has shown to achieve a complete remission in 21% and partial remission in 47% of treatment resistant disease, respectfully [31]. This observation is consistent with our *in vitro* analysis as documented. Hence, the characterization of biologic effects including modifications in apoptosis regulatory proteins not only solidifies proof of principle for treatment, but it also provides a further opportunity to outline and understand pathway for why partial remission or failure in BTK inhibition occurs in subpopulation of MCL patients.

The Bcl-2 family of proteins is essential for programmed cell death or apoptosis during development, tissue turnover and host defense against pathogens. The members of this family such as Bcl-2, Bcl-xL or Mcl-1 are altered in response to cell death inducers [19]. Here, we showed that treatment of MCL cells with Ibrutinib significantly decreased the expression of Bcl-2, Bcl-xL or Mcl-1 anti-apoptotic protein in Mino cells, but the inhibition of these proteins by Ibrutinib was modest in JeKo-1 cells. These finding indicate that the distinct inhibition of these anti-apoptotic proteins by Ibrutinib may explain why Mino and JeKo-1 cells differentially responded to Ibrutinib. Taken together, our findings suggest that the activation of BTK by overexpression or by phosphorylation or by both mechanisms plays a significant role in MCL cell survival and BTK may be a key target toward the treatment of patients harboring MCL.

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