Bruton's tyrosine kinase inhibitor restrains Wnt signaling in chronic lymphocytic leukemia

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Abstract. The B-cell receptor (BCR) signaling pathway serves an important role in the pathogenesis of chronic lymphocytic leukemia (CLL), and has been identified as a novel and effective therapeutic target of CLL, with particular focus its kinase factor, BTK. Previous studies have focused on combining the BTK inhibitor with additional chemotherapeutic agents to improve the prognosis of patients with CLL. Further investigation into the mechanism of the BTK inhibitor would promote an understanding of the pathogenesis of CLL. The current study investigated the association between ibrutinib and the Wnt signaling pathway, additionally focussing upon one of its regulators, metadherin (MTDH), which has been identified to be overexpressed in CLL and is considered a promoter of the Wnt pathway. The experiments in the current study were performed in the MEC-1 CLL cell line. Results indicated that MTDH, β-catenin and lymphoid-enhancing factor-1 were inhibited subsequent to ibrutinib treatment. The results indicate that in CLL, ibrutinib is likely to possess an inhibitory role in Wnt signaling.

Introduction

Chronic lymphocytic leukemia (CLL) is a disease that results from apoptotic resistance and an aberration in the accumulation of mature monoclonal B-cells. The prognosis of CLL has been greatly improved by the application of a combined regimen of fludarabine, cyclophosphamide and rituximab (FCR). However, there are certain patients with relapsed/refractory CLL who do not benefit from FCR treatment (1,2). Patients who exhibit refractory disease or a poor prognosis require novel therapeutic strategies.

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Genetic deficiencies, dysregulation of environmental factors and signaling pathways are involved in the pathogenesis of CLL (3-5). Among them, dysregulation of the B-cell receptor (BCR) signaling pathway is regarded as the most important mechanism, in addition to signals from the microenviroment, including interleukin 6, B-cell activating factor and CXCL12/stromal cell-derived factor 1 (6). Certain inhibitors of critical kinases involved in the BCR signaling pathway, including Bruton's tyrosine kinase (BTK), spleen tyrosine kinase and phosphoinositide 3-kinase (PI3K) have been suggested to improve the prognosis of patients with CLL (7). Previously, application of BTK inhibitors in CLL therapy has suggested that BTK may be a therapeutic target in CLL. It has been reported that patients with CLL that is previously untreated, has relapsed or is refractory can obtain benefits from ibrutinib therapy (8). In the NCCN Guidelines of 2004, the use of BTK inhibitors was listed in the treatment of CLL (9). Previous studies have focused upon the combination treatment of ibrutinib with other chemoimmunotherapeutic agents (10,11). In CLL, BCR signaling has been demonstrated to be associated with the PI3K, mitogen-activated protein kinase (MAPK) and nuclear factor κB pathways (12). However, the specific mechanisms involved in the use of BTK inhibitors in CLL require further elucidation.

In normal B-cells, BTK has been demonstrated to be a negative regulator of the Wnt signaling pathway by regulating CDC73 expression, however does not affect β-catenin expression (13). Another study on multiple myeloma indicated that BTK is able to regulate activity of Wnt by regulating CDC73, β-catenin, phosphorylated (p-) protein kinase B and p-glycogen synthase kinase 3β (GSK3β) (14). Considering the alterations to expression of Wnt signaling-associated molecules in tumors originating from B lymphocytes, in addition to the complex regulation of signaling pathways, it was suggested that investigation of the role of the BTK inhibitor in regulation of Wnt pathway of CLL may be beneficial.

Materials and methods

Cell culture and treatment reagents. The human MEC-1 CLL cell line was used in the current study. Cells were suspended in Iscove's modified Dulbecco's medium (IMDM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (GE Healthcare Life Sciences, Logan, UT,

USA) cultured at 37˚C with 5% carbon dioxide. BTK inhibitor (ibrutinib) were purchased from Selleck Chemicals (#S2680; Shanghai, China) and dissolved in dimethyl sulfoxide (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) to a concentration of 5 mM/l.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total mRNA of samples was isolated using TRIzol (Takara Biotechnology Co., Ltd., Dalian, China). Subsequently, the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) was used to measure the optical density (OD) value and the concentration of RNA. The OD 260/280 values of all RNA samples were read using the NanoDrop 2000 spectrophotometer (ratio, 1.8-2.0). The reverse transcription system was comprised of reverse transcription reagents, including 5X gDNA Eraser Buffer, gDNA Eraser , 5X PrimeScript Buffer 2 (for qPCR), RT Primer Mix, PrimeScript RT Enzyme Mix 1 and RNase Free dH_2O from Takara Biotechnology Co., Ltd. SYBR green-based RT-qPCR was performed following according to the manufacturer's instructions (Takara Biotechnology Co., Ltd.). The reaction system consisted of 10.0 μl SYBR Premix Ex Taq™, 0.4 μl PCR forward primer (10 μM) and 0.4 μl PCR reverse primer (10 μM), 2.0 μl cDNA and 7.2 μl distilled water. The RT-qPCR reaction was performed using the LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland). The primers used in the current study were as previously described (15,16). The sequences used were as follows: Metadherin (MTDH), F 5'-TTACCACCGAGC AACTTACAAC-3' and R 5'-ATTCCAGCCTCCTCCATT GAC-3'; lymphoid-enhancing factor 1 (LEF-1), F 5'-GAC GAGATGATCCCCTTCAA-3' and R 5'-AGGGCTCCT GAGAGGTTTGT-3'; β-catenin, F 5'-TGGCAGCAACAGT CTTACCT-3' and R 5'-CATAGCAGCTCGTACCCTCT-3'; and β-actin, F 5'-TGACGTGGACATCCGCAAAG-3' and R 5'-CTGGAAGGTGGACAGCGAGG-3'. Following preamplification (95˚C for 5 min), the PCRs were amplified for 40 cycles (95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 10 sec) on the LightCycler 480. The data were analyzed using LightCycler 480 software, version 1.5 (Roche Diagnostics), using the $2^{-\Delta\Delta Cq}$ method (17).

Protein isolation and western blot analysis. Total proteins were collected from the cells using radioimmunoprecipitation assay buffer and 1% phenylmethylsulfonyl fluoride (Shenergy Biocolor Bioscience & Technology Co., Ltd., Shanghai, China). The protein concentrations were measured using the Bicinchoninic Acid assay (Shenergy Biocolor Bioscience & Technology Co., Ltd.). Proteins were electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide gels (Sangon Biotech Co., Ltd., Shanghai, China), and were then transferred onto immobilon-P polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) . Membranes were then blocked for 1 h with Tris-buffered saline (Sangon Biotech Co., Ltd.) containing 5% non-fat dried milk and 0.1% Tween-20 (Solarbio Science & Technology Co., Ltd.). Subsequent to incubation with primary antibodies at 4˚C overnight, the membranes were incubated with anti-rabbit or anti-mouse IgGs conjugated to horseradish peroxidase (1:5,000; #ZB-2301 or #ZB-2305; OriGene Technologies, Inc., Beijing, China). The proteins of interest were detected with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (EMD Millipore). Primary antibodies were diluted using primary antibody dilution buffer (Beyotime Institute of Biotechnology, Shanghai, China) and secondary antibodies were diluted using secondary antibody dilution buffer (Beyotime Institute of Biotechnology). The antibodies used in the current study were as follows: Rabbit polyclonal anti-MTDH antibody (#13860-1-AP; 1:1,000; Proteintech Group, Inc., Chicago, IL, USA), rabbit monoclonal anti-β-catenin (#8480; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal anti-LEF-1 (#14972-1-AP; 1:1,000; Proteintech Group, Inc.), mouse anti-human monoclonal β-actin antibody (1:2,000) (#TA-09, OriGene Technologies, Inc., Beijing, China).

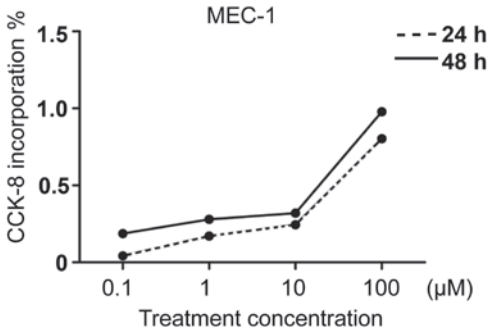

Figure 1. Proliferation inhibition of ibrutinib on MEC-1 cells. MEC-1 cells were incubated with or without increasing concentrations of PCI-32765 (0.1-100 μM) for 24 and 48 h. The CCK-8 assay was conducted to determine the viability of MEC-1 cells. Each experiment was repeated three times. CCK-8, Cell Counting Kit-8.

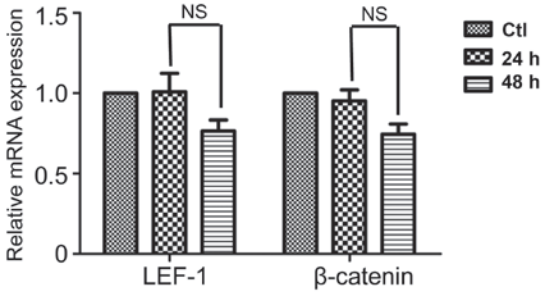

Figure 2. LEF-1 and β-catenin mRNA expression levels subsequent to ibrutinib treatment for 24 or 48 h ($^{NS}P>0.05$). LEF-1, lymphoid-enhancing factor 1; NS, non-significant; Ctl, control.

Cell proliferation. The cell suspension (10^4 cells) in 100 μl IMDM culture medium were plated into 96-well plates. Prior to the detection of proliferation, 10 μl Cell Counting Kit-8 solution (Nanjing EnoGene Biotech Co., Ltd., Nanjing, China) was added to each well, then the wells were cultured for 2 h at 37˚C. Subsequently, a SpectraMax M2 microplate reader (Molecular Devices, LLP, Sunnyvale, CA, USA) was used to detect the absorbance at a wavelength of 450 nm, which was adjusted at 690 nm.

Statistics analysis. SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis.

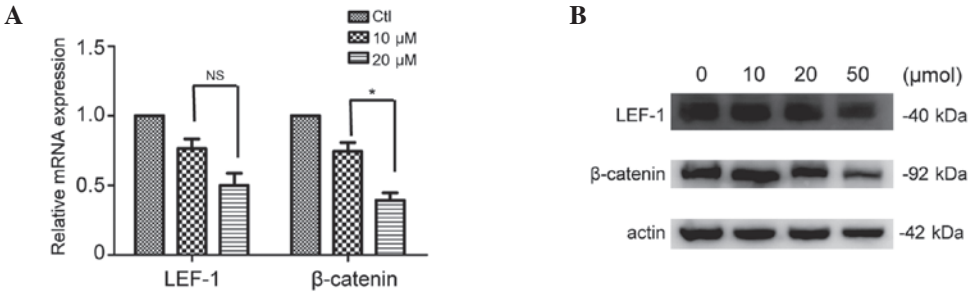

Figure 3. LEF-1 and β-catenin (A) mRNA and (B) protein expression levels subsequent to ibrutinib treatment at the indicated concentrations (^{NS}P>0.05, *P<0.05). LEF-1, lymphoid-enhancing factor 1; NS, non-significant; Ctl, control.

Figure 4. Downregulation of MTDH expression in MEC-1 cells subsequent to ibrutinib treatment (*P<0.05). MTDH, metadherin.

Student's t-test was used, and P<0.05 was considered to indicate a statistically significant difference.

Results

Ibrutinib inhibits the proliferation of MEC-1. Ibrutinib has been previously identified to exhibit a time- and concentration-dependent repressive effect on primary B-cells in CLL (18). In addition, the same study identified no significant difference in the inhibition ratio in CLL cells from patients with unmutated-immunoglobulin heavy chain variable (IgVH) or mutated-IgVH (18). In the current study, the effects of ibrutinib on the MEC-1 cell line were investigated. According to the methods of a previous study, the proliferation inhibition ratio was detected following ibrutinib treatment with different concentrations (0, 0.1, 1, 10, 100 μmol) (Fig. 1). The results indicated that in line with increases in treatment concentration of the BTK inhibitor, the proliferation of MEC-1 was inhibited compared with the control group. The proliferation inhibition was more marked in MEC-1 cells with treatment for 48 h compared with 24 h; however, no significant difference was identified between the two groups.

Ibrutinib inhibits the Wnt signaling pathway. Susbequent to treatment with ibrutinib for 48 h, the mRNA and protein expression levels of LEF-1 and β-catenin were measured in order to evaluate the activation state of Wnt signaling. The effects of ibrutinib on LEF-1 and β-catenin mRNA expression subsequent to treatment with 10 μM ibrutinib for 24 and 48 h were evaluated. The results indicated that there was a marginal time-dependent inhibitory effect of ibrutinib on LEF-1 and β-catenin expression, however this was not statistically significant between the two groups (P>0.05; Fig. 2). Accordingly, 48 h was selected for further study. The results demonstrated that subsequent to treatment with ibrutinib, the mRNA and protein expression levels of LEF-1 (non-significant) and β-catenin (P<0.05) were downregulated in a concentration-dependent manner (Fig. 3). The results indicate that inhibition of BCR signaling activity with ibrutinib in CLL may downregulate the activity of Wnt signaling in CLL.

Ibrutinib inhibits MTDH expression. In a previous study, it was demonstrated that MTDH is aberrantly overexpressed in CLL (19). Subsequent to interfering with MTDH expression in CLL, the downregulation of LEF-1 expression was detected, which was identified to be correlated with the downstream factors c-Myc and cyclin D1 (19). To detect whether the BTK inhibitor would inhibit CLL cell viability via inhibition of MTDH expression, mRNA and protein MTDH expression levels prior and subsequent to 48 h ibrutinib treatment were measured. The results indicated that subsequent to ibrutinib treatment, MTDH was downregulated, and the inhibitory rate was associated with the concentration of ibrutinib (P<0.05; Fig. 4).

Discussion

As one of the most important signaling pathway in CLL, BCR signaling is central to the development and maintenance of B-cell malignancies. The BCR consists of immunoglobulin heavy and light chains coupled to a CD79A-CD79B heterodimer that transduces signals by engaging downstream nonreceptor kinases, including BTK (12). BTK is a member of the Tec family of kinases and is involved in the transduction of signals in BCR-signaling and the mediation of B-cell activation. Ibrutinib, which has undergone clinical trials, has been demonstrated to exhibit clinical efficacy and safety in numerous B-cell malignancies, including mantle cell

lymphoma (MCL), diffuse large B-cell lymphoma, follicular lymphoma and CLL, as a single agent in addition to use in combination therapy (8,10,11,20,21).

Ibrutinib is an irreversible BTK inhibitor that has been previously described as a covalent inhibitor of BTK able to bind at cysteine 481 (22). Ibrutinib was approved by the Food and Drug Administration in November 2013 for the treatment of relapsed MCL and in February 2014 for relapsed/refractory CLL (23). It is suggested that ibrutinib may be used in the future to complement traditional immunotherapy. In addition, it has been demonstrated that ibrutinib possesses potential to eliminate the need for chemoimmunotherapy (8,10). An improved understanding of the mechanisms of ibrutinib will aid in the development of effective combination therapy strategies to improve the prognosis of patients with CLL (24,25). It has been reported that *in vitro*, apoptosis can be induced in primary CLL cells by administration of a high concentration of ibrutinib (18). In the current study, the CLL cell line MEC-1 was treated with ibrutinib, in order to identify the optimal culture conditions for the subsequent experiments. The results indicated that the activity of ibrutinib in MEC-1 cells was time- and concentration-dependent. For the further evaluation of the effect of ibrutinib, 48 h was selected for the following experiments. Subsequently, the effect of ibrutinib on the Wnt signaling pathway in CLL was evaluated.

The canonical Wnt signaling pathway is associated with a variety of cellular processes and is involved in carcinogenesis (26-28). Its activation is mediated by the inhibition of the activity of GSK3β, and leads to the stabilization of β-catenin, thus promoting nuclear translocation. In the nucleus, β-catenin interacts with DNA-binding protein T-cell factor/lymphoid-enhancing factor family members to drive the transcription of various target genes including cyclin D1 and c-Myc (29). Wnt signaling-associated genes including WNT-3, WNT4, WNT-5B, WNT-7B, WNT-9A, WNT-10A, WNT-16B and LEF-1 have been demonstrated to be overexpressed in CLL (16,30,31). Previous studies have demonstrated that inhibition of the WNT signaling pathway by small-interfering RNA of LEF-1 or inhibitors such as CGP049090 and PKF115-584 can effectively reduce the proliferation of CLL and induce apoptosis of CLL cells (32,33). This indicates that the Wnt signaling pathway may be a potential therapeutic target in CLL (16,33-35).

BTK, which has been previously demonstrated to be a downregulator of the Wnt signaling pathway in normal B-cells, can also promote the activity of the Wnt pathway in multiple myeloma (14,15). The current study aimed to investigate the association between ibrutinib and the Wnt signaling pathway. In the current study, LEF-1 and β-catenin were selected for the evaluation of the function of Wnt signaling. The results indicated that subsequent to treatment with ibrutinib for 48 h, LEF-1 and β-catenin were correspondingly inhibited. This indicated that BTK may promote the activity of the Wnt pathway in CLL.

In a previous study, it was demonstrated that over-activation of the Wnt signaling pathway is partially associated with overexpression of MTDH (36). With the inhibition of MTDH expression, CLL cells were identified to exhibit apoptosis in addition to downregulation of Wnt signaling pathway molecules, including LEF-1, with no clear downregulation of β-catenin in the levels of total protein (19). Under conditions of BCR activation, MTDH has previously been identified to be upregulated in CLL cells. (36). In addition, it has been observed that LEF-1 is upregulated in line with increases of MTDH (data not shown).

Whether inhibition of BTK expression would affect MTDH expression was investigated in CLL in the current study. A downregulation in the MTDH mRNA and protein expression levels was identified in MEC-1 cells subsequent to treatment with ibrutinib for 48 h. These results indicate that BTK is an upstream regulator in CLL, and that its inhibition leads to the downregulation of MTDH. Further studies should focus upon elucidating the precise mechanisms of the regulatory role of BTK in MTDH and LEF-1 regulation. This would aid in increasing the understanding of the pathogenesis of CLL.

Multiple signaling pathways have been identified to be dysregulated in CLL, including the Wnt, Notch, BCR, MAPK and PI3Kδ pathways. The complex regulation of different signaling pathways is key in the molecular mechanisms of refractory and chemoresistant tumors. Further investigation into the regulatory mechanisms of signaling in CLL and other tumors would aid in improving understanding of the pathogenesis of these diseases, thus benefiting the development of effective therapeutative strategies.

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