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Bruton’s tyrosine kinase: potential target in human multiple myeloma

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Abstract
Bruton’s tyrosine kinase (BTK), a Tec family non-receptor tyrosine kinase that is required for B cell development, is critical for the initiation and maintenance of human B-cell malignancies. However, the expression of BTK and the role that BTK plays in the pathogenesis of multiple myeloma (MM) remain seldom reported. In this study we examined the expression and screened for gene mutations of BTK in MM cells. We showed that BTK was elevated and activated in a dexamethasone-resistant cell line and in two out of nine (22.2%) patients’ cells. Interestingly, patients with higher BTK expression had a poorer prognosis. In addition, a single nucleotide polymorphism (SNP) at cDNA position 2062 (T2062C) in the BTK gene was recorded in six out of eight (75%) patients and in U266 cells. This SNP in MM cells was not detected in other malignant hematopoietic cells of different lineages. These results suggest that the function of BTK warrants further investigation, and BTK expression might be used as a prognostic indicator for patients with MM.

Keywords: BTK, multiple myeloma, patients, prognostic indicators

Introduction
Multiple myeloma (MM) is a B-cell malignancy with terminally differentiated plasma cell phenotype, which accounts for 10% of all hematologic cancers and approximately 2% of all cancer deaths [1]. It is characterized by clonal expansion of bone marrow (BM) plasma cells that produce monoclonal immunoglobulin. The signaling pathways leading to tumorigenesis and etiology are not fully explained [2–4].

Given the importance of tyrosine kinase activation in a variety of human malignancies [5], receptor and non-receptor tyrosine kinases have emerged as targets in tumor therapy. The Tec family formed by BTK, BMX, ITK, TEC and RLK are the second largest group of non-receptor tyrosine kinases [6]. Functionally, Tec kinases play pivotal roles in the development and signaling of hematopoietic cells [7–9]. Bruton’s tyrosine kinase (BTK) is by far the most studied member of the Tec family. BTK regulates many vital signal pathways including phosphatidylinositol-3-kinase (PI3K), phosphoinositide phospholipase γ (PLCγ) and protein kinase C (PKC). These pathways play critical functions in cell proliferation, development, differentiation, survival and apoptosis [10]. Mutations in BTK result in the B-cell immunodeficiencies X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (xid) in mice [11,12]. These diseases are characterized by blocks in B-cell development at multiple stages and impaired function of residual mature B-cells. BTK is mainly expressed in B cells, and is also expressed in myeloid, mast cells [13]. BTK is expressed throughout the development of B cells and is not expressed in T cells and other non-hematopoietic cell lineages [14]. However, there are few reports about the expression of BTK in malignant plasma cells and the function it plays in plasma cell neoplasms. Recently, Tai et al. [13] used gene expression profiling to show robust BTK expression in malignant plasma cells from the majority of patients with MM (> 85%), and proposed it as a novel target in the bone marrow microenvironment.

To obtain a comprehensive insight into the function of BTK in MM, the aims of the present research were to examine the expression of BTK, screen BTK gene mutation in MM and investigate the relationship of BTK expression to prognosis. Our findings suggest that BTK is expressed on the mRNA and protein levels and is activated in MM. Interestingly, dexamethasone-sensitive (MM.1S) and dexamethasone-resistant (MM.1R) human MM cell lines had notably different protein expression and phosphoryla-
tion levels. In addition, two out nine (22.2%) patients with MM had relatively high $BTK$ mRNA expression levels compared with U266 and RPMI 8226 cell lines. Further, we investigated the clinical background of the two patients who had higher expression levels of $BTK$, and found that several prognostic indicators were poorer than in other patients. Furthermore, we screened for variations of the coding sequence of the $BTK$ gene by cDNA sequencing in MM cell lines and eight patients with MM. The results showed that U266 cells and six out eight patients (75%) carried a single nucleotide polymorphism (SNP) at cDNA position 2062 (T2062C) in the $BTK$ gene. Although this SNP does not alter the coding amino acid of the codon, it appears to be common in MM, and possibly MM-specific. Therefore, the function of $BTK$ in MM warrants further investigation.

Materials and methods

Patients

MM patient-samples were obtained from the Department of Oncology, the Second Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou and Department of Hematology, Nanfang Hospital Affiliated to Nanfang Medical University, Guangzhou after informed consent. Nine patients were studied. The diagnosis was based on clinical data and examination of the bone marrow according to the French–American–British classification. CD138+ cells from nine patients with MM were isolated, with informed consent, from BM mononuclear cells using positive immunomagnetic column separation (Miltenyi Biotech, Auburn, CA). The purity of CD138+ cells was above 97% as determined by flow cytometry [15].

Cells and reagents

The human MM cell lines U266 and RPMI 8226, the pro-myelocytic cell line HL-60, the human leukemic monocyte lymphoma cell line U937, and the erythro-leukemia cell line K562 were purchased from the American Type Culture Collection (ATCC). Dexamethasone-sensitive (MM.1S) and dexamethasone-resistant (MM.1R) human MM cell lines were kindly provided by Professor Jie Jin (Department of Hematology, the First Affiliated Hospital, Zhejiang University College of Medicine). Raji Burkitt lymphoma cells and T cell line Molt-4 were kindly provided by Dr. Hong-Qian Zhu from the Department of Hematology, Nanfang Hospital. All cell lines were incubated in a humidified CO$_2$ incubator (5% CO$_2$, 37°C).

Real-time quantitative PCR

Expression of the $BTK$ gene was examined by real-time polymerase chain reaction (PCR) normalized to the expression of $\beta$-actin. Total RNA was extracted from cell lines or patients’ cells using TRIzol reagent (Invitrogen). For real-time PCR, we used $BTK$ gene forward primer 5’-GAG AAGCTGTTGCAATGTAT-3’, $BTK$ gene reverse primer 5’-GG CGGAATCATGACCTTAA-3’, and $\beta$-actin forward primer 5’-CTCTCTGAGGGCGAAGTAC-3’, $\beta$-actin reverse primer 5’-TCTTGTTGATCCACAT-3’.

Western blot

Cell pellets were lysed and protein extracts were quantitated and loaded on 8% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed and transferred to a nitrocellulose membrane (Whatman). The membrane was incubated with primary antibody, washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce). Detection was performed using a chemiluminescent Western detection kit (Cell Signaling Technology). Antibodies used included: anti-$BTK$ (Santa Cruz), anti-phospho-$BTK$ (Y223) (Epitomics) and anti-\(\beta\)-actin (Sigma-Aldrich).

Statistical analysis

All experiments were repeated at least three times, and data are presented as mean ± SD unless noted otherwise. Data were analyzed using the software SPSS 12.0 for Windows (Chicago, IL).

Results

$BTK$ is expressed in MM and in myeloid cell lines

$BTK$ expression was studied by real-time quantitative PCR and Western blot in MM cell lines U266 and RPMI 8226, and four myeloid cell lines U937, HL-60, K562 and Kasumi-1. The Burkitt lymphoma line Raji (human B-cell line) was used as a positive control, and T-cell line Molt-4 as a negative control. Initial results showed that $BTK$ was expressed in MM cell lines at the mRNA level. Interestingly, the MM cell line RPMI 8226 and U266 which produces interleukin-6 (IL-6)-producing cell line U266 had notably different mRNA expression levels [Figure 1(A)]. In addition, the protein level of $BTK$ was detected by Western blot; results showed that the expression of $BTK$ was detectable [Figure 1(B)]. To further assess the activation of $BTK$, phospho-$BTK$ (Y223) antibody was used for Western blotting. The results showed that phosphorylation of $BTK$ was also detectable in these cell lines, correlating with the results of protein expression [Figure 1(C)]. We further investigated the expression of $BTK$ and phosphorylation of $BTK$ in dexamethasone-sensitive, MM.1S, and dexamethasone-resistant, MM.1R, human MM cell lines, and found that $BTK$ had higher expression and phosphorylation in MM.1R than in MM.1S cells [Figures 1(D) and 1(E)]. A previous article reported that $BTK$ was involved in imatinib resistance in chronic myeloid leukemia [16]. Altogether, these data indicate that $BTK$ is expressed in both MM and myeloid cell lines at the protein and mRNA levels. The higher expression and phosphorylation of $BTK$ in dexamethasone-resistant cells may contribute to chemoresistance.

$BTK$ is expressed in patients with MM and higher

$BTK$ may be associated with poor prognosis

Since the MM cell lines RPMI 8226 and U266 showed $BTK$ expression, we next sought to investigate whether patients with MM displayed a similar effect. $BTK$ expression was investigated by real-time PCR in nine patients (Figure 2). U266 and Molt-4 were used as positive and negative controls of the different expression levels. The results indicated that $BTK$ had a relatively high expression level in two of nine (22.2%) patients. Patient 1 and patient 3 had high
expression levels compared with U266 and RPMI 8226. We investigated the clinical background of the nine patients (Table I), and found that patients 1 and 3 had a poorer prognosis compared with the other patients, including more neoplastic cells in the bone marrow milieu (>30%), elevated levels of β2-microglobulin (β2-M, >3.5 mg/L) and age >60 years. In addition, hemoglobin, platelet and albumin levels were lower in the two patients, again associated with a poor prognosis. In conclusion, BTK gene expression was detected by real-time quantitative PCR in nine patients, and two patients (numbers 1 and 3) had relatively high levels of BTK mRNA expression. The elevated expression of BTK may have a correlation with poor prognosis.

Screening for SNP in BTK gene cDNA of MM cells
Since BTK plays a role in XLA, we aimed to screen for variations of coding sequence of the BTK gene by cDNA sequencing in MM cell lines and eight unrelated patients. The sequencing results showed that the U266 cell line and six out of eight patients (75%) carried a SNP at position 2062 (T2062C) in cDNA of BTK coding region. However, the alteration did not change the coding amino acid of the codon [Figures 3(A) and 3(B)]. To find whether this SNP is characteristic of MM cells, sequences of the BTK gene in four myeloid cell lines (U937, HL-60, K562 and Kasumi-1) and Raji were screened. The sequencing results showed that the SNP did not exist in these cell lines [Figure 3(C)]. These results suggest that the T2062C SNP may be specific to MM cells. This SNP at position 2062 in cDNA of BTK coding region, which plays a critical role in BTK activation. Based on these results, it could be hypothesized that the T2062C SNP may be associated with an increased risk of BTK activation.

Discussion
In this study, we have provided evidence that BTK is expressed in human multiple myeloma cells at both the mRNA and the protein level. Previous reports have indicated that the BTK gene is expressed at high levels in cells of B lymphocyte and myeloid lineages and also in a mast cell leukemia line, whereas low or undetectable levels were found in T cells and plasma cells [14]. Smith et al., using Northern blotting, confirmed that BTK transcripts were undetectable in four MM lines, U266, U1996, L363 and Karpas 707. Conversely, Tai et al. [13], using gene expression profiling, showed robust BTK expression in malignant plasma cells from the majority of patients with MM (>85%), and suggested it as a novel target in the bone marrow microenvironment. Using real-time PCR and Western blot, we also demonstrated that BTK is expressed and activated in four MM cell lines, U266, RPMI 8226, MM.1S and MM.1R. Interestingly, BTK had higher expression and activation in MM.1R than in MM.1S cells; this means that BTK may play a critical role in chemoresistance. Further, the BTK gene was expressed in almost all of the obtained MM
patient-samples. In this study, we also showed that MM cell lines U266 and RPMI 8226 exhibited dramatically different expression levels. Using these different expression levels as a standard, we determined BTK expression in patient cells. The results suggested that two out of nine patients had relatively higher expression of BTK compared with U266 and RPMI 8226. In addition, we found that the two patients with higher expression of BTK had a poorer prognosis, including a higher neoplastic cell count in the bone marrow milieu, elevated levels of $\beta_2$-M, higher age and lower hemoglobin, platelet and albumin levels, compared to the other seven patients. The investigation of prognostic indicators may contribute to the identification of risk categories and preparation of more accurate information regarding individual disease outcome. Here the BTK expression pattern may help to establish the foundation for a future, novel prognostic indicator of MM.

We screened for variations of the coding sequence of the BTK gene by cDNA sequencing and found a SNP at position 2062 (T2062C) in the U266 cell line and six out of eight patients (75%). Also, the SNP did not exist in the other hematopoietic malignant cells (U937, HL-60, K562 and Kasumi-1) and Raji cells. These results suggest that the T2062C SNP may not be a general feature, but may occur only in MM subtypes. In addition, this SNP in cDNA of BTK coding region, which plays a critical role in BTK activation. Therefore, we hypothesized that the T2062C SNP may be associated with an increased risk of BTK activation.

An oral BTK inhibitor, ibrutinib, with excellent pharmacodynamics, has achieved high response rates in phase I/II clinical trials in relapsed non-Hodgkin lymphoma, and phase III clinical trials in chronic lymphocytic leukemia (CLL) and mantle cell lymphoma. Due to its lower toxicity and efficacy, this agent has great promise, either as a single agent or in combination treatment, in other B cell malignancies as well [17]. The therapeutic potential of targeting BTK or upstream/downstream effectors associated with BTK has been proposed and reviewed by several researchers [18,19]. The anti-apoptosis function of BTK is supported by the observation that bcl-2 and bcl-xl transgenes can restore normal B-cell levels in xid mice [20]. Consistent with the anti-apoptotic function of BTK, treatment of leukemic cells with a BTK inhibitor enhanced their sensitivity to chemotherapy-induced apoptosis [21]. Hence, the expression pattern of BTK may help establish the foundation for a future, new prognostic factor of MM, and the therapeutic potential of targeting BTK may warrant further investigation.

**Potential conflict of interest:** Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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References


