# Bortezomib Therapeutic Effect Is Associated with Expression and Mutation of *FGFR3* in Human Lymphoma Cells

WEIYAN ZHENG[1,2*], MIN GUAN[2*], LIJUN ZHU[3], ZHEN CAI[1], VINCENT CHUNG[4], HE HUANG[1] and YUN YEN[2]

[1]*Department of Hematology, Bone Marrow Transplantation Center, the First Affiliated Hospital, Zhejiang University, School of Medicine, Hangzhou, Zhejiang, 310003, P.R. China;*
[2]*Department of Molecular Pharmacology, Beckman Research Institute, and*
[4]*Department of Medical Oncology and Therapeutic Research, City of Hope National Medical Center, Duarte, CA, 91010, U.S.A;*
[3]*Department of Basic Medical Sciences, Zhejiang University School of Medicine, Hangzhou, Zhejiang, 310058, P.R. China*

**Abstract.** *Objective: Ectopically expressed and deregulated fibroblast growth factor receptor 3 (FGFR3) has been observed in many malignant cancer patients, including those with lymphoma. This study investigated whether the therapeutic effect of bortezomib in lymphoma is associated with FGFR3-expression. Materials and Methods: Cell proliferation and apoptosis assays were performed in minimal FGFR3 expressing U937 cells and compared to U937 cells overexpressing FGFR3 wild-type, or Y373C or K650E mutant FGFR3. Results: Results from this study suggested the expression of FGFR3 protein is associated with the therapeutic effect of bortezomib. It was observed that bortezomib-induced apoptotic death is correlated with FGFR3 expression. U937 cells overexpression of wild-type FGFR3 demonstrated resistance to bortezomib treatment. U937 cells expressing Y373C mutated FGFR3 showed an almost equal resistance to bortezomib as U937 cells expressing wild-type FGFR3. U937 cells expressing mutated K650E FGFR3 showed more sensitivity to bortezomib than did the parental U937 cells. Furthermore, increased expression of Mcl-1 and decreased expression of NF-κB p65 suggested that bortezomib resistance associated with Y373C mutation and wild-type FGFR3 may be partly mediated through Bcl-2 and NF-κB signaling. Conclusion: Data from this study indicate that mutation status and the expression level of FGFR3 may be associated with bortezomib-related treatment resistance in lymphoma.*

Fibroblast growth factor receptor 3 (FGFR3) has been found to be up-regulated in several type of cancer, including lymphoma (1, 2). FGFR3 promotes cancer cell growth by three general mechanisms: by autoendosis as mitogens for cancer cells themselves, by promoting angiogenesis and inhibiting apoptosis (1).

Overexpression of FGFR3 has been reported in Hodgkin's lymphoma (HD) and T-cell lymphoma (2-4). However, few lymphoma patients have chromosomal translocation t(4;14)(p16.3;q32.3) which results in overexpression of FGFR3 (5). Highly constitutive expression of FGFR3 has been found in Hodgkin's cell lines and in Hodgkin and Reed-Sternberg (H/RS) cells of HD patients but not in reactive lymphoid cells (6). This may be explained by some somatic activating mutations of *FGFR3* in HD (6). Activating mutations of *FGFR3* are observed frequently in human bladder and cervical carcinomas (7). The activating mutation K650E, when present in the germ line, causes thanatophoric dysplasia type II (TDII) (8). Another kinase-activating mutation, Y373C, in the transmembrane domain of FGFR3, and an unknown functional mutation, G384D, have been identified in multiple myeloma (MM) patients (7).

Bortezomib, the first proteasome inhibitor approved by Food and Drug Administration of the United States (US FDA), has been used successfully for the treatment of relapsed or refractory MM and mantle cell lymphoma (MCL)

*Both authors contributed equally to this work.

*Correspondence to:* Dr. Yun Yen, Department of Molecular Pharmacology, Beckman Research Institute, City of Hope, CA 91006, U.S.A. Tel: +626 2564673, Fax: +626 4713607, e-mail: yyen@coh.org / Dr. He Huang, Department of Hematology, Bone Marrow Transplantation Center, the First Affiliated Hospital, Zhejiang University, School of Medicaine, Hangzhou, Zhejiang, 310003, P.R. China. Tel: +86 57188208009, Fax: +86 57187236707, e-mail: hehuangyu@126.com

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(9). *In vitro* and *in vivo* studies have demonstrated single-agent activity against various lymphoid tumors and additive or synergistic effects in combination with other agents including standard chemotherapy employed in non-Hodgkin's lymphoma (NHL) (10). Phase II clinical trials indicate that bortezomib is well tolerated and active in several NHL subtypes, with response rates of 18-60% in follicular lymphoma (FL) and 39-56% in MCL (11-14).

Bortezomib affects various growth and survival pathways in cancer cells, including: i) inhibition of the adhesion of cancer cells, resulting in blockade of the adhesion-related transcription and secretion of multiple cytokines; ii) inhibition of nuclear factor kappaB (NF-κB); iii) impairment of the DNA repair machinery; iv) down-regulation of growth and anti-apoptotic signaling pathways and associated proteins (14-17). The most important mechanism involved in lymphoma treatment is the regulation of the Bcl-2 apoptotic gene family and transcription factor NF-κB (15).

It has been reported that the effect of bortezomib on cancer cell growth and survival is associated with FGFR3 expression in MM (18). In this study, the most common hematological malignancy, lymphoma, was explored in order to test whether FGFR3 expression associated with the bortezomib therapeutic effect is cell line independent. In order to investigate the efficiency of bortezomib in lymphoma cells *in vitro* and determine whether bortezomib therapeutic resistance is associated with deregulation of FGFR3 expression, this study aimed to test the effect of bortezomib in lymphoma cells overexpressing wild-type, Y373C or K650E mutant FGFR3 *via* transfection of designated plasmids into the parental cells, as well as in parental cells lacking expression of FGFR3.

## Materials and Methods

*Expression vector construction*. A vector containing full-length human *FGFR3* cDNA was donated by Dr. Podolsky (Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA). To remove the 3' untranslated region, a *Hind III*/*Kpn I* fragment was released from the vector containing full-length FGFR3 cDNA. This fragment was subsequently ligated into a *Hind III*/*Kpn I*-digested pcDNA3.0 plasmid (Invitrogen, San Diego, CA, USA). Y373C and K650E mutations were created with QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, CA, USA) (Y373C 5' primer GCGGGCAGTGTGTGTGCAGGCATCCTC; 3' primer GAGGAT GCCTGCACACAC ACTGCCCGC; K650E 5' primer CTCGACT ACTACAAGGAGACAACCAACGGCC; 3' primer GGCCGTTG GTTGTCTCCTTGTAGTAGTCGAG) (Figure 1). Mutations were confirmed by DNA sequencing.

*Cell culture and transfection*. U937 human lymphoma cell line, which lacks FGFR3, was obtained from the American Tissue Culture Collection (ATCC). Cells were cultured in RPMI1640 medium (Cellgro, Mediatech Inc., VA, USA), supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin and streptomycin (100 μg/ml) and maintained at 37˚C in a 5% $CO_2$ atmosphere. U937 cells ($2\times10^6$) were transfected using Nucleofector Device and reagent (Amaxa, USA) with 2 μg of each plasmid containing full-length human cDNA encoding wild-type *FGFR3*, Y373C or K650E mutations. Stable transfectants were established by selection in a medium supplemented with 800 μg/ml G418 (Life Technologies, CA, USA.) for 3 weeks. These clones were designated Y-U937, K-U937 and T-U937, expressing the Y373C, K650E mutant and wild-type FGFR3, respectively.

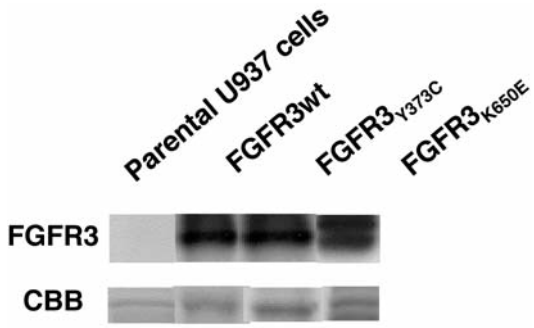


Figure 1. *Overexpression of wild-type, Y373C or K650E FGFR3 in FGFR3-deficient lymphoma U937 cells. U937 cells lacking endogenous FGFR3 were transfected using electroporation with pcDNA 3.1 plasmid containing full-length human cDNA encoding FGFR3. Western blot analysis revealed the expression of FGFR3 protein in transfected U937 cells. Alpha-tubulin was used as loading control.*

*RT-PCR*. Total RNA was extracted from the cells using RNeasy Mini kits (Qiagen, Germany) according to the instructions of the manufacturer. Total RNA was reverse transcribed and amplified using superscript one-step reverse transcriptase-PCR (RT-PCR) system with a platinum Taq polymerase (Invitrogen, Life Technologies). The FGFR3-specific primers (forward primer 5'-TGCTGAATGCCTCCCACG-3', reverse primer 5'-CGTCTTCGTCATCTCCCGAG-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers (forward primer 5'-CCACATCGCTCAGACACCAT-3', reverse primer 5'-CCAGGCGCCCAATACG-3') were employed for amplification of *FGFR3* and *GAPDH*. To avoid the background of products amplified from genomic DNAs, primers for detecting expressions of *FGFR3* and *GAPDH* were designed to exist on two different exons. *FGFR3* and *GAPDH* were amplified as follows: 1 cycle of 50˚C for 30 minutes, 94˚C for 2 minutes, 25 cycles of denaturing at 94˚C for 30 seconds, annealing at 53˚C for 45 seconds, and extending at 72˚C for 1 minute, followed by a final elongation step at 72˚C for 10 minutes.

*Bortezomib*. Bortezomib, obtained from Dr. Vincent Chung (City of Hope, Duarte, CA, USA), was dissolved in DMSO and stored at –80˚C. Bortezomib was diluted in cultured medium ($10^{-4}$-$10^{-2}$ μmol/l) with <0.1% DMSO immediately before use.

*$IC_{50}$ determination in parental cells and transfectants*. Cells were seeded into 96-well microculture plates at 20,000 cells/well overnight before treatment with bortezomib at a dose range of 0.001-10 μM for 24 hours. Cell survival/cytostasis was then quantified using the tetrazolium dye MTS as previously described (18). Each experimental data point represents average values obtained from eight replicates, and each experiment was performed in triplicate. Viability measurements were confirmed by trypan blue exclusion.

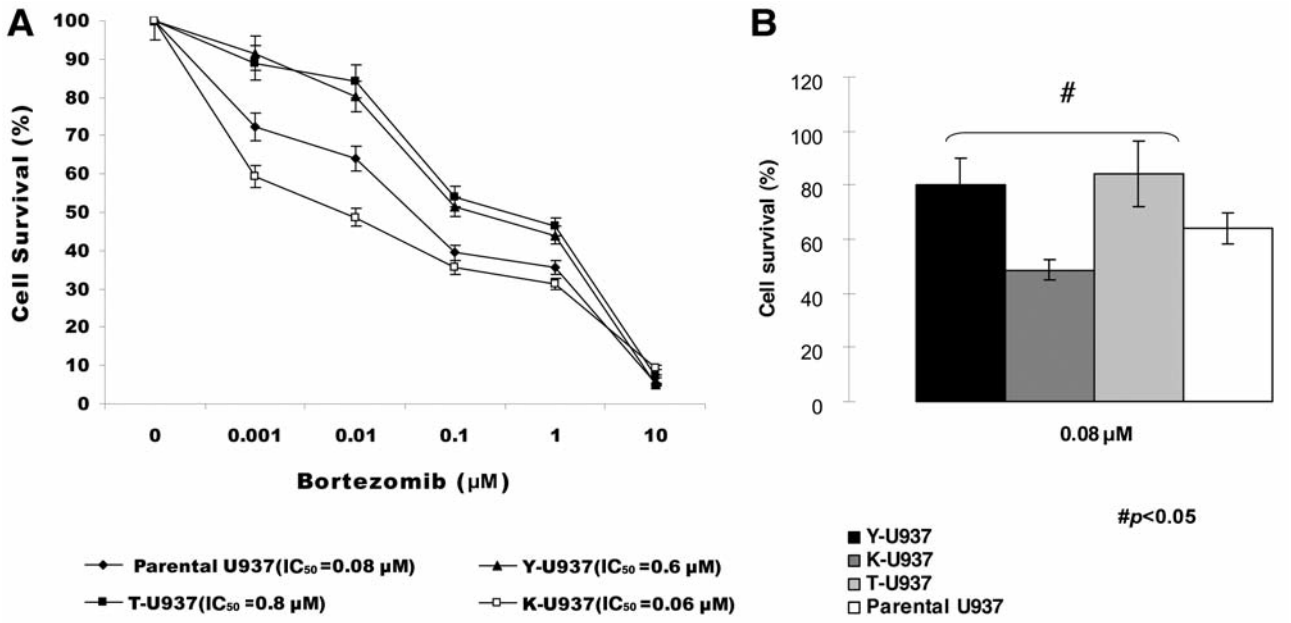


Figure 2. *A: $IC_{50}$ determination in parental cells and FGFR3 transfectants. $IC_{50}$ was determined in parental and transfectant cells by treating the cells with a dose range from 0.001 to 10 μM of bortezomib diluted with DMSO. Twenty-four hours later, cell survival was examined by MTS incorporation. Data represent means and SDs for triplicate experiments. B: Drug resistance to bortezomib in Y-U937 and T-U937 transfectants. Bortezomib-induced U937 cell death was determined by MTS assay at 24 hours post treatment at 0.08 μM. Data are shown as a percentage when compared to the DMSO-treated cells as a control (100%). Each experiment was performed in triplicate. Results are presented as mean±SD. #p<0.05, t-test. T-U937 and Y-U937 cells were compared with K-U937 and parental U937 cells.*

*Cell proliferation and Annexin V apoptosis assay.* Cells were seeded into 96-well microculture plates at 20,000 cells /well and incubated with bortezomib for 24 hours. At the end of treatment, 20 μl of CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA) was added into each well containing 100 μl of cell suspension. The plate was incubated for 4 hours at 37˚C then the absorbance of soluble formazan produced by cellular reduction of MTS was measured at a wavelength of 490 nm. Values for the experimental conditions were normalized to a control value of 1 for each experiment. Assays were reported as the average of at least three separate experiments.

A total of $1\times10^5$ cells were rinsed and resuspended in binding buffer 24 h after treatment with 1 nM bortezomib. Then 5 μl of Annexin V (BD Biosciences Clontech, Palo Alto, CA, USA) and 10 μl of propidium iodide were added to the cell suspension and incubated at room temperature for 15 min in the dark. Cells were analyzed using a single laser emitting light at 488 nm on the NoFlo MLS flow cytometer at the Analytical Cytometry Core (City of Hope, Duarte, CA, USA).

*Antibodies and Western blot analysis.* Human anti-VEGF polycolonal antibody was obtained from ZYMED (San Francisco, CA, USA) and anti-FGFR3 polyclonal antibody, which recognizes the *C*-terminal FGFR3 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for human STAT3, Mcl-1, Raf, Erk and phosphorylated Erk were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cells were harvested, washed with ice-cold PBS, and lysed in RIPA buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1%

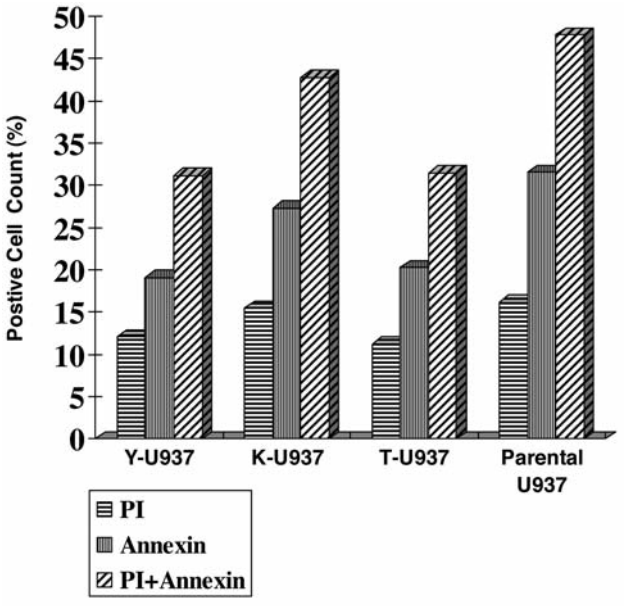


Figure 3. *Bortezomib-induced apoptosis is associated with FGFR3 expression level. Bortezomib induced apoptosis in U937 cells was determined through Annexin V- FITC staining by flow cytometry at 24 hours' post treatment at 0.001 μM. The number of positive-staining cells were significantly increased in K-U937 and parental U937 when compared with Y-U937 and T-U937 cells.*

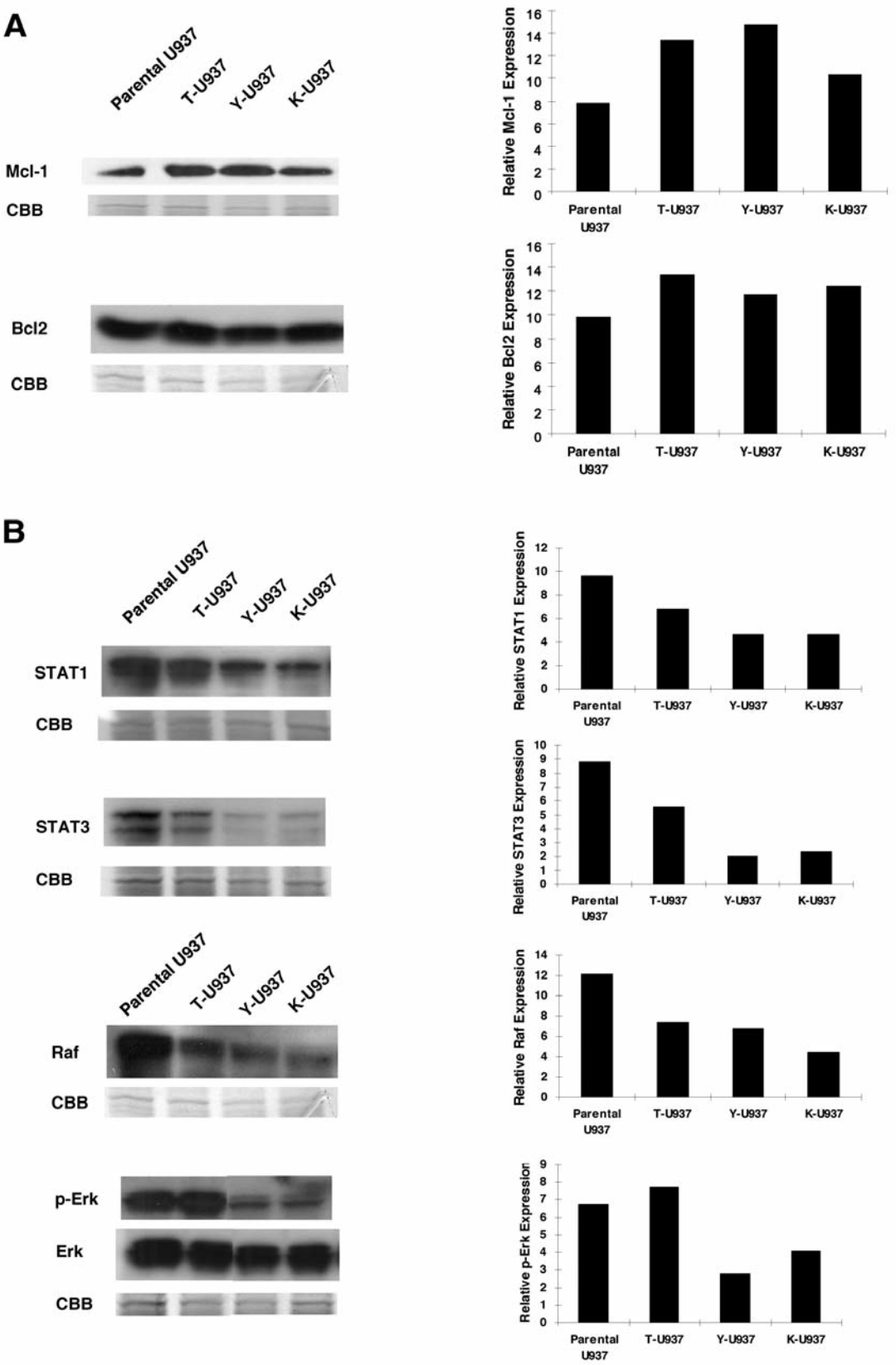


Figure 4. *continued*

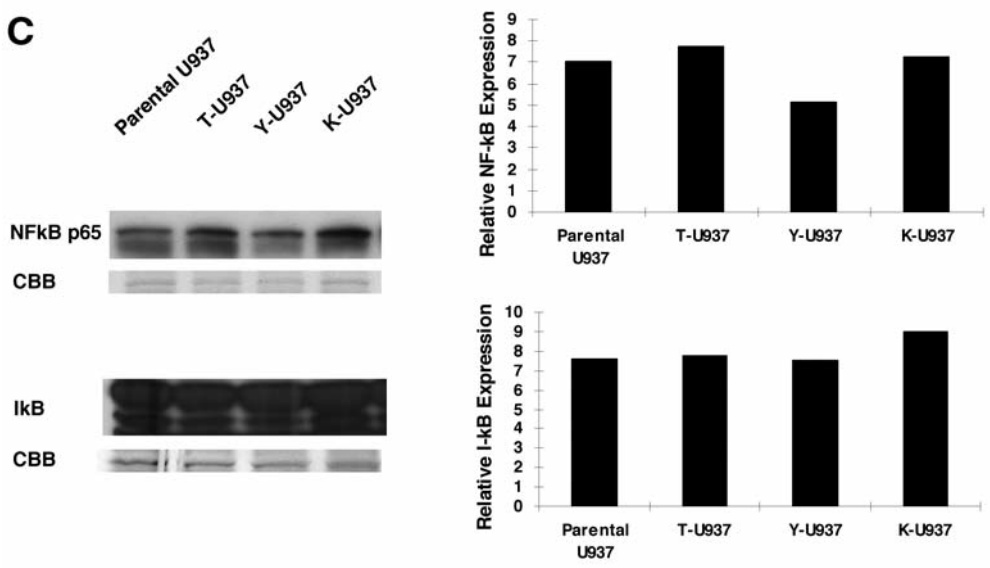


Figure 4. *A: Increased expression of Mcl-1 and Bcl2 in all FGFR3 transfectants. B: Decreased expression of STAT1, STAT3, Raf and p-Erk in Y-U937 and K-U937 cells. C: Decreased expression of NF-κB in Y-U937 cells. All proteins were examined by Western blot, CBB was used as control for protein loading. Quantification of proteins was performed by Image Quant 2.5 and corrected relative to the control.*

SDS) with freshly added protein inhibitors (100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, and 30 μl/ml aprotinin). The samples were separated on 7.5% SDS-polyacrylamide gel by loading equivalent amounts of protein. After electrophoresis, the proteins were transferred to a PVDF membrane (Amersham Pharmacia Biotech, Sweden). The membrane was incubated with antibodies under the manufacturer's Western blot protocol. Immunoblots were detected by the Western-Light™ System (Applied Biosystems) Foster City, CA, USA). PVDF membranes were stripped and reprobed with actin as loading control. Values of the relative amounts of protein were determined by the densities of the bands by ImageQuant version 5.2 (Molecular Dynamics, Sunnyvale, CA, USA).

*Statistical analysis*. All experiments were repeated at least three times and each sample in at least triplicate for each experiment. The values reported the mean of triplicate (±SD). Student's *t*-test was used to examine the significance of cell survival and apoptosis assay in transfectant and parental cells.

## Results

*$IC_{50}$ determination in parental cell and FGFR3 transfectants*. The lymphoma cell line U937 used in this study was negative for t(4;14) and any endogenous FGFR3. U937 cells were transfected with the plasmid encoding a full-length *FGFR3* cDNA of wild-type or Y373C or K650E mutations (T-U937, Y-U937 and K-U937, respectively). Stably transfected clones were selected and expression of FGFR3 was determined by both mRNA and protein levels as previously described (18). FGFR3 was not detected by RT-PCR and Western Blot in parental U937 cells. However, high levels of FGFR3 were detected in all transfectants, indicating that overexpression of FGFR3 can be achieved in *FGFR3*-deficient cells through plasmid transfection. Expression of FGFR3 was identified in all constructs (Figure 1).

To determine the $IC_{50}$ of bortezomib, different transfectants, as well as the parental U937 cells, were treated with bortezomib at the dose range of 0.001-10 μM for 24 hours, before performing the cell proliferation assay by MTS incorporation as previously described (18). As shown in Figure 2A, cell survival decreased in a dose-dependent manner after treatment with bortezomib in transfectant and parental cells. The parental U-937 and K-U937 cell survival declined more rapidly after exposure to bortezomib than did that of the Y-U937 and T-U937 cells. K-U937 appears to be extremely sensitive when exposed to bortezomib. Complete cell death was observed at a dose of ≥10 μM in all cell lines (data not shown). The $IC_{50}$ of bortezomib for Y-U937, T-U937, K-U937 and parental U937 cell lines was approximately 0.6, 0.8, 0.06 and 0.08 μM, respectively, which was statistically significant ($p \leq 0.01$).

*Y-U937 and T-U937 cells demonstrated resistance to bortezomib*. To determine whether FGFR3 affects the sensitivity of bortezomib in malignant lymphoma, cell proliferation by MTS incorporation was performed in different transfectants as well as the parental U937 cells. As shown in Figure 2B, about 20% of Y-U937 cells and 16% of T-U937 cells died after exposure to 0.08 μM bortezomib for 24 hours as compared to control cells, whereas 51% of

K-U937 cells and 36% of parental U937 cells died under similar conditions. These results indicated that the FGFR3-transfected T-U937 and Y-U937 cells are more resistant to bortezomib than the parental U937 cells, although K-U937 cells remain the most sensitive to bortezomib as compared to the parental U937 cells, indicating a significant drug-resistant phenotype in Y-U937 cells *versus* a sensitive phenotype observed in K-U937 cells.

*Bortezomib-induced apoptosis is associated with FGFR3 expression level*. To determine whether apoptotic cell death is induced by bortezomib, the Annexin V–FITC apoptosis assay was performed as previously described (18) in *FGFR3* transfectants and parental cells. Apoptotic cells were detected by flow cytometry after incubation with 0.001 μM bortezomib for 24 hours. As shown in Figure 3, exposure of Y-U937 and T-U937 cells to bortezomib resulted in only 19% and 12% cells undergoing apoptosis. Moderate apoptosis was observed with K-U937 and parental U937 cells (21% and 27% apoptosis, respectively) ($p \leq 0.05$). These findings again suggest that Y-U937 and T-U937 are resistant to bortezomib-mediated apoptosis.

*FGFR3 is associated with Mcl-1 and NF-κB signaling pathway*. To examine the possible signaling pathways mediated by FGFR3, the signal pathway factor Raf and related downstream targets (p-Erk, STAT1 and STAT3, Bcl-2 family and NF-κB family) were also analyzed from cell extracts by Western blot analysis. The quantity for each protein was determined (Image Quant 5.2) and presented in Figure 4, which has been corrected for loading.

Interestingly, one of the important pro-apoptotic members in the Bcl-2 family, Mcl-1, was increased in all three transfected cell types, but weakest in K-U937. Other Bcl-2 family members including Bclx1 were not changed (data not shown). Since the Raf-Mek-Erk kinase cascade is an important signaling pathway of tumor cell survival and apoptosis, Western blot analysis was performed using several antibodies that bind to total Raf, phosphorylated Erk and total Erk. As shown in Figure 4, total Raf was decreased in all *FGFR3* transfectants, especially in K-U937 and Y-U937 cells. A similar phenomenon was observed for p-Erk but not for total Erk expression; the latter was almost unaffected in all *FGFR3* transfectants as compared to the level in parental U937 cells.

Total STAT1 and STAT3 in transfectants with FGFR3 were significantly reduced when compared to parental U937. In T-U937 transfectant, Raf and downstream factors STAT1 and STAT3 decreased, but Erk and p-Erk did not change significantly. Most importantly, the anti-apoptosis factor Mcl-1 increased significantly, and this may play a crucial role in the resistance to bortezomib. In Y-U937 and K-U937 transfectants, Raf, Erk, STAT1 and SATA3 decreased rapidly compared to parental U937. Up-regulation of anti-apoptosis gene *Mcl-1* synergized with down-regulation of NF-κB p65, resulting in resistance to bortezomib in Y-U937. However, a significantly increased expression of NF-κB p65 in K-U937 cells counteracted the mildly increased expression of Mcl-1; this led to increased sensitivity of K-U937 cells to bortezomib compared to parental U937 cells.

## Discussion

Proteasome inhibitor bortezomib has been proven as an effective drug in treating relapsed/refractory multiple myeloma and certain subtypes of NHL. In the present and a previous study (18), both lymphoma (U937) and myeloma cells (U266) have been found to be highly sensitive to bortezomib *in vitro*.

Bortezomib activity has been demonstrated in phase I and II studies on relapsed or refractory indolent lymphomas. O'Connor *et al*. demonstrated an overall response rate (ORR) of 58%, with an ORR of 50% in MCL and 77% in FL (17). The activity in NHL has been confirmed by other phase II trials, with response rates of 18-60% in FL and 39-56% in MCL (11-13). The PINNACLE trial treated 141 relapsed/refractory MCL patients with 1.3 mg/m$^2$ of bortezomib twice weekly, demonstrating an ORR of 31% with a median duration of response of 9.2 months (19). Combinations with standard agents are performed in several treatment centers and confirm a significant improvement. Barr *et al*. (20) studied a phase I trial of fludarabine, rituximab combination with bortezomib for relapsed and refractory indolent and mantle cell NHL. In that trial, from a total of 24 patients, clinical responses were observed in 11, 5 of whom were refractory to their most recent treatment regimen. Six additional patients had stable disease for a median of 10 months (range 4–30+). A randomized phase II trial from the French Adult Lymphoma Study Group (GELA) showed that R-CHOP plus bortezomib was an effective regimen in treating CD20 positive B-cell lymphoma, and produced an 84% CR rate (21).

This study showed that wild-type *FGFR3* transfected U937 and U266 cell survival increased 20-30% on bortezomib treatment as compared to the parental cells. This result reveals FGFR3 as one of the key factors in bortezomib resistance. As regards FGFR3-activating mutation, Y373C and K650E took a completely different mechanism and activation. The Y373C mutation caused a strong resistance to bortezomib, but K650E mutation resulted in a most sensitive reaction to bortezomib in both lymphoma and myeloma cell lines.

Overexpression or activity mutation of FGFR3 is well established in myeloma development and progression (18). The present data showed that FGFR3 had a similar behavior in the lymphoma cell line, but possibly through different mechanisms.

Several mechanisms have been proposed to explain the antitumor activity of bortezomib: i) NF-κB inhibition; ii) reduction of antiapoptotic (Bcl-2) and angiogenic factors; iii) stabilization and accumulation of p53; iv) deregulation of cyclin turnover; v) shift in the balance between pro- and anti-apoptotic Bcl-2 family proteins; vi) deregulated proapoptotic signaling *via* TNF-related apoptosis-inducing ligand (TRAIL); vii) disruption of the unfolded protein response with endoplasmic reticulum (ER) stress induction and viii) reversal of IL-6- and IGF-1-mediated antiapoptotic effects (14-17). However, the most important factors in the treatment of lymphoma are the NF-κB transcription factors and Bcl-2 apoptotic family. FL is characterized by the t(14;18) translocation, which results in overexpression of the antiapoptotic protein Bcl-2 (22-23). NF-κB is constitutively active in FL; extranodal MZL may harbor t(1;14), t(11;18), and t(14;18) translocations, which can lead to NF-κB activation (22, 23). This may explain why bortezomib is more sensitive in treating subtypes of lymphoma than MM.

NF-κB transcription factors are central regulators in lymphocyte proliferation, survival and development, and are recognized as key pathological feature in various lymphoid malignancies. Constitutive activation of NF-κB has been detected in HD cells (24). Proteasome inhibitor triggered H/RS cell apoptosis is associated with a down-regulation of NF-κB target genes and reduced tumor growth *in vivo* (24). Davis *et al*. demonstrated a significant up regulation of NF-κB target genes CCND2, IRF4, CFLAR, CCR7 and NFKBIA in some subtypes of B cell lymphoma (*e.g*. activated B cell like diffuse large B cell lymphoma (ABC-DLBCL), primary mediastinal B cell lymphoma (PMBL)) by microarray gene expression analysis (25). Since NF-κB is required for the survival of various lymphoma cells, inhibition of NF-κB may be an attractive therapeutic approach in malignant lymphoma. Previous research has shown that the activation of NF-κB p65 signaling in MCL is stronger than that in MM (26).

In contrast, the apoptosis gene family Bcl-2 undergoes a crucial mechanism in lymphoma pathogenesis. Bcl-2 family comprises three functionally distinct types of proteins. Proapoptotic Bak and Bax can form multisubunit complexes that compromise the integrity of the outer mitochondrial membrane (27). This leads to cytochrome-*c* release, assembly of the proapoptotic molecule APAF-1, and activation of executioner caspases. Antiapoptotic proteins, such as Bcl-2, Bcl-Xl, and Mcl-1 antagonize this process by binding to and neutralizing Bak and Bax. In turn, these antiapoptotic proteins can be titrated by a third class of Bcl-2 protein, the proapoptotic BH3-only proteins such as Bim and NOXA (28). Bortezomib inhibits proteasome activity leading to the accumulation of apoptosis protein and inducing tumor cell death. Ri *et al*. found that bortezomib-induced apoptosis in mature T-cell lymphoma cells partially depends on up-regulation of NOXA and functional repression of Mcl-1 (29).

These results suggest that signal transformation and regulation mechanisms may be different in the three *FGFR3* transfectants (T-U937, Y-U937, K-U937). For T-U937, STAT3-Mcl-1 signaling pathway may play a crucial role in the resistance to bortezomib. For Y-U937, rapid inhibition of the Raf-Erk kinase cascade (phosphorylated Erk declined to a very low level) may lead to anti-apoptosis in lymphoma cells. Y-U937 expresses significantly low levels of NF-κB p65, which is the key molecule in bortezomib therapy. Thus, low levels of NF-κB in Y-U937 synergizing with low expression of Raf and ERK may result in the resistance to bortezomib. In contrast, K-U937 expresses NF-κB p65 at a notably high level, which may counteract the inhibition of Raf-ERK and lead to the sensitivity to bortezomib. Other apoptosis and angiogenesis molecules such as p21, AKT, cyclin and cdk2 were examined and no significant changes were seen (data not shown).

Resistance to bortezomib remains a challenge in lymphoma treatment. It is reported that bortezomib resistance occurs in more than 50% of the recurrent/relapse lymphoma patients (30). Previous data have shown that wild-type FGFR3 and Y373C mutations are associated with bortezomib resistance in MM (18). This study has found that bortezomib therapeutic resistance is also associated with deregulating FGFR3 expression in lymphoma cells, but possibly through different signaling pathways.

Statistically, 10-25% of MM patients have been observed to have ectopically expressed FGFR3, resulting from the chromosomal translocation t(4; 14)(p16.3; q32.3) (5). Kinase-activating mutations Y373C and K650E have been identified in MM patients (30, 31). Joos *et al*. (6) detected increased copy numbers of the *FGFR3* gene due to translocated telomeric chromosomal segments in two out of four HD cell lines, although only few cases were reported in lymphoma patients with t(4;14)(p16.3;q32.3). However, some somatic activating mutation of FGFR3 is present in HD (34). Yagasaki *et al*. (35) reported a peripheral T-cell lymphoma patient with a t(4;12)(p16.3;p13) chromosomal translocation, which resulted in fusion of *ETV6* to *FGFR3*. Khnykin *et al*. (2) showed high expression of FGFs and their receptors (FGFR1-4) in H/RS cells of HD, but not in reactive lymphoid cells. Few data have been published to date involving FGFR3 expression levels in NHL.

FGFR3 is a membrane-spanning tyrosine kinase receptor that has a high affinity for FGFs. FGFR3 contains three glycosylated extracellular immunoglobulin-like domains, a transmembrane domain, and a split intracellular tyrosine kinase domain. Under ligand stimulation, FGFR3 undergoes dimerization and tyrosine autophosphorylation, resulting in cell proliferation or differentiation (5, 7, 8, 31).

FGFR3 signals influence a variety of cellular events and processes, largely through inducing or repressing expression of target genes in a cell-specific context. Four main signaling pathways have been identified to date to propagate FGFR3 signals: STAT, MAPK, PLC-g, and PI3K-AKT (1-5). STAT1 signals are thought to induce expression of mitotic inhibitors, such as the cdk inhibitor p21 (34). Using microarrays to assess changes in gene expression in chondrocytic cells, Dailey *et al*. (35) showed that FGFs initiate signals in multiple pathways that result in the induction of antiproliferative functions and down-regulation of growth-promoting molecules. A kinase-activating mutation Y373C activates FGFR3 by promoting constitutive dimerization in the absence of the ligand. Dimerization activates the intrinsic tyrosine kinase activity of the receptor and promotes transphosphorylation of key tyrosine residues in the cytoplasmic domain. These residues serve as docking sites for adapter proteins and signal effectors that are recruited to the activated receptors and which propagate FGFR3 signals (5, 7, 8, 31). Monsonego-Ornan *et al*. (36) suggested that this mutation slows receptor internalization, leaving it on the surface to signal. The free cysteine residues introduced by the mutation are believed to form disulfide bonds resulting in dimerization, which in turn activates the receptor (37). K650E mutation is identified in a split cytoplasmic tyrosine kinase domain. This mutation alters the conformation of the kinase domain, constitutively activating the intrinsic enzyme activity (5, 7, 8, 31).

This study showed that transfected lymphoma U937 cells with wild-type, Y373C or K650E activate different signal pathways. The opposite changes in Raf-Mek-Erk and STAT1 and STAT3 were observed in U937 lymphoma cell lines in comparison to the MM cells. One explanation may be that the Raf-Mek-Erk pathway and STAT1 and STAT3 cascades in lymphoma cells are excessively activated prior to *FGFR3* transfection, which could explain the change caused by FGFR3. Another explanation may due to the cell origin differing in MM and lymphoma, making the FGFR3 downstream pathways different (38). This study showed that Bcl-2 family, NF-κB, Raf-Mek-Erk, STAT1 and STAT3 pathways were changed distinctly, though in different trend and grade.

Ronchetti *et al*. (7) found that *FGFR3* mutations are graded in terms of their activation capability. Under serum-starved conditions, *FGFR3* mutation cell lines express appreciable levels of phosphorylated mutant FGFR3. The addition of a FGF ligand increased the level of receptor phosphorylation further. Howerever ligand-stimulated *FGFR3* mutants activated the MAP kinase signaling pathway but did not apparently involve either the STAT1 or STAT3 cascades. When transfected in 293T cells, Y373C and K650E were able to activate MAPK, STAT1 and STAT3 under serum-starved condition.

Zhu *et al*. (39) demonstrated that antiapoptotic genes *Bcl-2, Mcl-1, CFLAR* and some members of the NF-κB family were down-regulated, whereas the expression of proapoptotic genes *CYC, BID, CASP2* and *CASP6* increased when shRNAs were used to inhibit *FGFR3* expression in Y373C or K650E mutant cell lines. *Ras* and *Raf*, the important genes in the Raf-Mek-Erk pathway, were also down-regulated. In this study, wild-type or mutant *FGFR3* genes were transfected into minimal FGFR3-expressing U937 cells. As expected, the antiapoptotic gene *Mcl-1* was distinctly up-regulated in all three transfected cell lines (T-U937, Y-U937, K-U937), but other Bcl-2 family members were not changed remarkably when compared to parental U937 cells. This situation may be attributed to the fact that Bcl-2 family is always strongly expressed in lymphoma cells, including the U937 cell line. So this minimum alteration cannot be observed clearly.

This study demonstrated that the increased expression of Mcl-1 in U937 cell lines transfected with *FGFR3* before bortezomib treatment may be the key point of the bortezomib resistance. Perez-Galan *et al*. (40) found that treatment of MCL with bortezomib led to an accumulation of Mcl-1, as well as increasing the level of the BH3-only protein NOXA. It has been suggested that the excess NOXA displaces Bax from Mcl-1, and free Bax then induces caspase activation, outweighing the anti-apoptotic effect of Mcl-1 (40). Other studies also indicate that bortezomib treatment results in accumulation of Mcl-1 and the cleaved form of Mcl-1 in T-cell lymphoma, MM and melanoma (41). Mcl-1 is an important regulator of apoptosis in response to cellular stress and to different DNA damaging agents, and is strictly regulated. It has a short half-life and is rapidly degraded by the proteasome (41). The accumulation of Mcl-1 may be due to the reduction of proteasomal degradation, and may affect bortezomib induced apoptosis. Pretreatment with Mcl-1-specific siRNA or other agents to repress Mcl-1 will consequently enhance bortezomib-triggered apoptosis in MM and melanoma cells (42). But if Mcl-1 increases before bortezomib treatment, it will affect the drug response strongly, and consequently induce drug resistance.

It is worthy of note that when transfected with Y373C mutant FGFR3, Y-U937 cells showed notably reduced NF-κB p65 before given bortezomib. It has reported that lower levels of NF-κB before treatment result in drug resistance of proteasome inhibitor. Bortezomib inhibits both the canonical and alternative NF-κB pathways by inhibiting proteasome mediated degradation of IkB proteins and processing of p100, respectively and has been proven by preclinical and clinical trials. The clinical effect of bortezomib is related to the high expression of NF-κB in lymphoma patients (24).

Indeed, owing to the heterogeneous biological activation of the diseases, the signaling pathways of FGFR3 are likely graded and varied. How the mechanism of action of bortezomib varies in different disease states is still unclear.

Taken together, these findings indicate the sensitivity of bortezomib in lymphoma treatment. However, *FGFR3* mutations or other undetermined mutations may allow for more useful bortezomib combinations in order to overcome treatment resistance.

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