Bortezomib Therapeutic Effect is Associated with Expression of FGFR3 in Multiple Myeloma Cells

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Abstract. *The ectopically expressed and deregulated fibroblast growth factor receptor 3 (FGFR3) has been observed in ~20% of multiple myeloma (MM) patients. In this study, we investigated whether the therapeutic effect of bortezomib is associated with FGFR3 expression. Materials and Methods: Cell proliferation and apoptosis assays were performed in minimal FGFR3 expressing U266 cells and compared to U266 cells overexpressing FGFR3 wild-type (T-U266), or Y373C (Y-U266) or K650E (K-U266) mutant FGFR3. Results: Our results suggested cell survival decreases in a dose-dependent manner. Interestingly, expression of FGFR3 protein was similarly dose dependent on bortezomib. It is confirmed the bortezomib-induced apoptotic death is correlated with FGFR3 expression. Furthermore, increased expression of p-STAT3, Mcl-1 and VEGF suggested that bortezomib resistance associated with Y373C mutation and wild-type FGFR3 may be partly mediated through p-STAT3 signaling. Conclusion: Our data indicates that Y373C mutation and wild-type FGFR3 may be associated with bortezomib-related treatment resistance in multiple myeloma.*

The chromosomal translocation t(4;14) (p16.3;q32.3) that results in overexpression of FGFR3 has been identified in approximately 10-25% of multiple myeloma (MM) patients (1). This translocation results in ectopic overexpression of functional FGFR3 and promotes myeloma cell proliferation and prevents apoptosis (2, 3). In some cases of MM, mostly in late-stage disease, the translocated *FGFR3* is mutated, which includes an activating mutation K650E that, when present in the germ line, causes thanatophoric dysplasia type II (TDII) (4). A kinase-activating mutation Y373C in the transmembrane domain of FGFR3 and an unknown functional mutation G384D have been also identified in MM patients. It should be noted that FGFR3 has also been found mutated in MM without t(4;14) translocation (5). Activating mutations of *FGFR3* are observed frequently in human bladder and cervical carcinomas (6, 7) and overexpression of FGFR3 has also been reported in Hodgkin's and T-cell lymphomas (8-10). In MM however, evidence suggests a pathological role for FGFR3 in disease development and progression, possibly due to the fact that FGFR3 is oncogenic when overexpressed and activated by FGF ligand or an active mutation (2).

Bortezomib was the first proteasome inhibitor to enter clinical trials based on results of preclinical studies showing that this novel agent directly inhibits the proliferation of myeloma cells, induces apoptosis and abrogates paracrine tumor growth (11, 12). Bortezomib affects various growth and survival pathways in MM cells including: inhibition of the adhesion of multiple myeloma cells to bone marrow stromal cells (BMSCs), resulting in blockade of the adhesion-related transcription and secretion of multiple cytokines; inhibition of NF-κB; impairment of the DNA repair machinery; down-regulation of growth and antiapoptotic signaling pathways and associated proteins, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), Akt, Bcl-2, Mcl-1, inhibitors of apoptosis proteins (IAPs), and growth factor receptors (13-16). It also inhibits VEGF (17-19) and reduces STAT activity (20). Several large phase II and III studies of bortezomib were conducted in patients with advanced relapsed and/or refractory multiple myeloma (MM) and

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lymphomas (9). Favorable results of these studies led to accelerated approval for use of bortezomib in MM patients who have progressed after at least their second therapy and, more recently, to expanded approval for second-line use in patients on whom one prior therapy has failed. However, prolonged exposure is associated with toxicity and eventual resistance (21, 22). In order to determine whether bortezomib resistance is associated with FGFR3 status and its signaling pathway, we set out to test the effect of bortezomib in MM cells overexpressing wild-type or Y373C and K650E mutant FGFR3 *via* transfection of designated *FGFR3* expression plasmids into the parental cells, as well as in MM cells lacking overexpression of FGFR3.

Materials and Methods

Expression vector construction. A vector containing full-length human *FGFR3* cDNA was a gift from Dr. Podolsky (Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, 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). Y373C and K650E mutations were created with QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, CA, USA) (Y373C 5' primer GCGGG CAGTGTGTGTGCAGG CATCCTC, 3' primer GAGGATGCCTGCA CACACACTGCCCGC; K650E 5' primer CTCGACTACTACAAGG AGACAACCAA CGGCC, 3' primer GGCCGTTGGTTGTCTCCTT GTAGTA GTCGAG) (Figure 1B). Mutations were confirmed by DNA sequencing.

Cell culture and transfection. U266 human multiple myeloma cell line, which lacks *FGFR3*, was obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI Type 1640 medium (Cellgro, Mediatech Inc., Herndon, 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. Two million U266 cells were transfected using Nuclefector Device and reagent (Amaxa, USA) with 2 μg of each plasmid containing full-length human cDNA encoding wild-type *FGFR3* and Y373C and K650E mutations. Stable transfectants were established by selection in medium supplemented with 800 μg/ml G418 (Life Technologies, Inc.) for 3 weeks. These clones were designated Y-U266, K-U266 and T-U266, which express the Y373C, K650E mutant and wild-type *FGFR3*, respectively.

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, CA). The *FGFR3*-specific primers (forward primer 5'-TGCTGAATGCCTCCCACG-3', reverse primer 5'-CGTCTT CGTCATCTCCCGAG-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, kindly provided by Dr. Chung (City of Hope, Duarte, CA, USA), was dissolved in DMSO and stored at –80˚C. Bortezomib was diluted in culture 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 0.0001-10 μM for 24 h. Cell survival/cytostasis was then quantified using the tetrazolium dye MTS. Each experimental data point represented average values obtained from eight replicates, and each experiment was performed in triplicate. Viability measurements were confirmed by trypan blue exclusion.

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 h. 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 absorbance of soluble formazan produced by cellular reduction of MTS was measured at the 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 1×10^5 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 was added to the cells suspension and incubated at RT 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 purchased from ZYMED (San Francisco, CA, USA) and anti-FGFR3 polyclonal antibody, which recognizes the *C*-terminal FGFR3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human STAT3, phosphorylated STAT3, Mcl-1 and VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz).

Cells were harvested, washed with ice-cold PBS, and lysed in RIPA buffer (1x PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% 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% sodium dodecyl sulfate-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 Western blot protocol from the manufacturer. Immunoblots were detected by the Western-Light™ System (Applied Biosystems. Foster City, CA, USA). PVDF membranes

A

Translocation and FGFR3 expression in multiple myeloma

Cell Line	t(4;14)	FGFR3	FGFR3 mut	nt change	N, K-ras mut	nt change
KMS-11	+	+++	Y373C	TAT>TGT	ND	-
OPM-2	+	++	K650E	AAG>GAG	ND	-
NCI-H929	+	+	ND	-	N13-G>D	GGT>GAT
U266	-	-	-	-	-	-

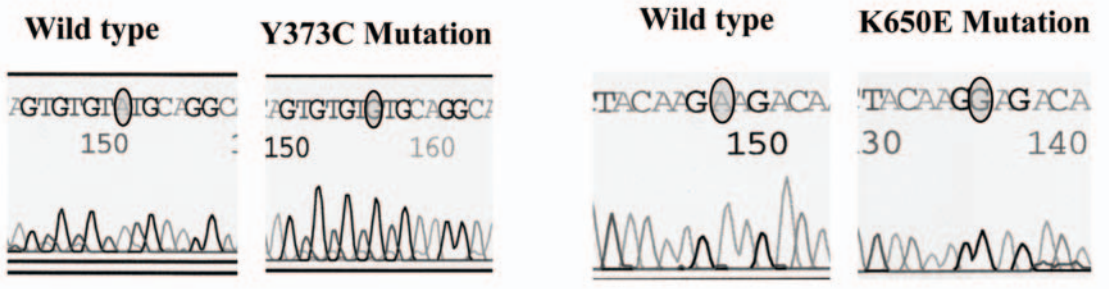
mut: Mutation, nt: nucleotide, ND: not detected.

B

Mutation primers

Y373C 5 GCGGGCAGTGTG*TGT*GCAGGCATCCTC
Y373C 3 GAGGATGCCTGC*ACA*CACACTGCCCGC
TAT->TGT

K650E 5 CTCGACTACTAC*AAG*GAGACAACCAACGGCC
K650E 3 GGCCGTTGGTTG*TCT*CCTTGTAGTAGTCGAG
AAG->GAG



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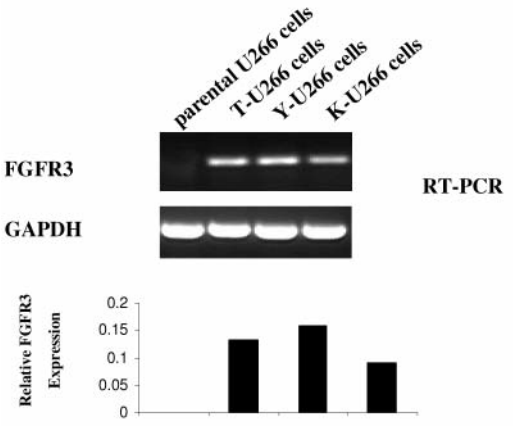

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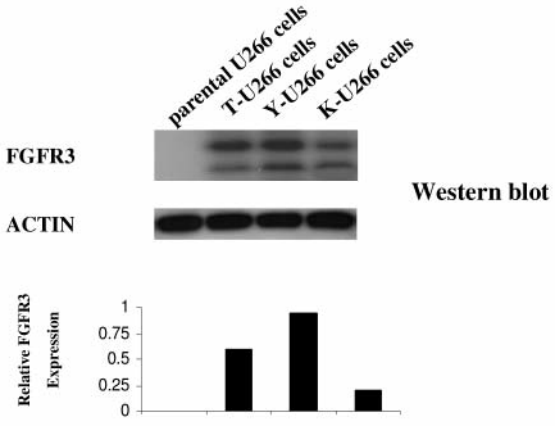

Figure 1. *Overexpression of Y373C and K650E mutant and wild-type FGFR3 in tranfected U266 cells. U266 cells lacking endogenous FGFR3 were transfected using electroporation with pcDNA3.1 plasmid containing full-length human cDNA3 encoding FGFR3. A, Translocation and FGFR3 expression in multiple myeloma. B, Site-mutated primers were designed to generate the single mutation Y373C or K650E by PCR. The sequencing result indicated the mutation was successfully introduced. C, RT-PCR analysis revealed the expression of FGFR3 in transfectant clones. The upper panels indicate mRNA extracted from the parental cells and transfectants. GAPDH was amplified as the internal control. D, Western blot analysis revealed the expression of FGFR3 protein in transfected U266 cells. Actin was used as loading control.*

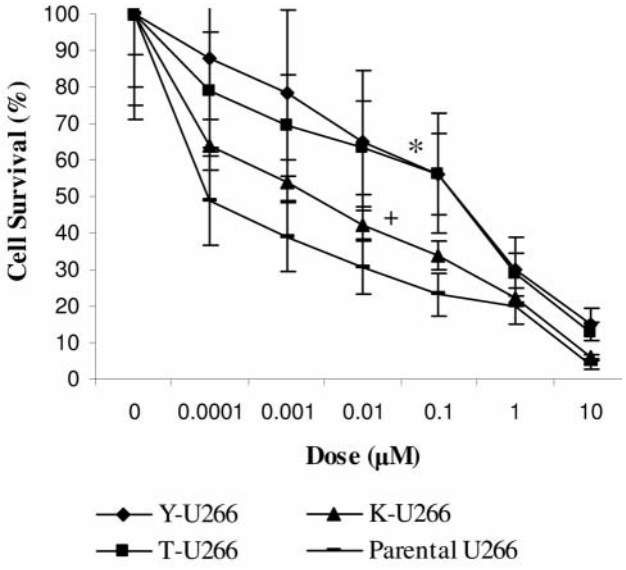

Figure 2. *IC_{50} determination in parental cells and FGFR3 transfectants. IC_{50} was determined in parental and transfectant cells by treating the cells with a dose range from 0.1 nM to 10 μM of bortezomib diluted with DMSO. Twenty-four hours later, cell survival was examined by MTS incorporation. Data represent means and SDs for triplicate experiments, *p<0.01, t-test, Y-U266 and T-U266 cells compared with parental cells; +p<0.05, t-test, K-U266 cells compared with parental cells.*

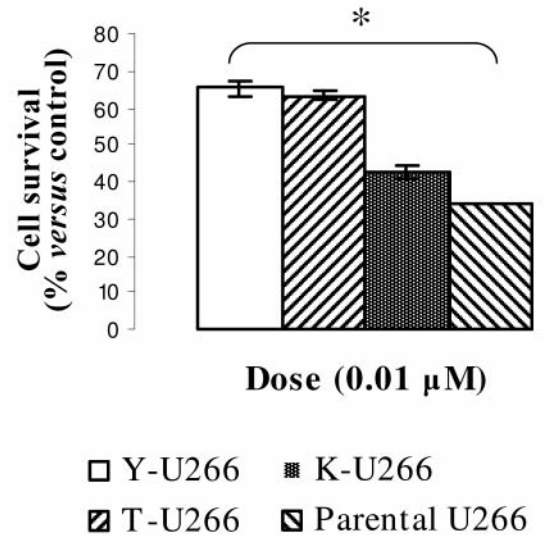

Figure 3. *Drug resistance to bortezomib in Y-U266 and T-U266 transfectants. Bortezomib-induced U266 cell death was determined by MTS assay at 24 hours post treatment at 0.01 μM. Data shown as a percentage when compared to the DMSO-treated cells as a control (100%). Each experiment was performed in triplicate. Results are presented as mean±SD. *p<0.01, t-test, Y-U266 and T-U266 cells compared with parental cells.*

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 ≥ triplicate in each experiment. The values reported the mean of triplicates (±SD). The *t*-test was used to examine the significance of cell survival and apoptosis assay in transfectant and parental cells.

Results

Overexpression of wild-type, Y373C and K650E FGFR3 in FGFR3-deficient MM U266 cells. The MM cell line U266 used in this study is negative for t(4;14) and any endogenous FGFR3 (Figure 1A). U266 cells were transfected with the plasmid encoding a full-length *FGFR3* cDNA of wild-type or Y373C or K650E mutations (T-U266, Y-U266 and K-U266, respectively). Stably transfected clones were selected after prolonged culture in selection medium. The expression of FGFR3 was determined by both mRNA and protein levels. As shown in Figure1C and D, FGFR3 cannot be detected by RT-PCR and Western blot in parental U266 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 (Figure1 C and D).

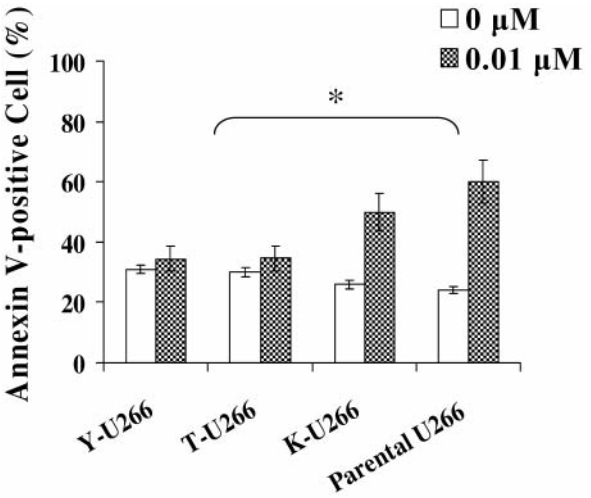

Figure 4. *Bortezomib-induced apoptosis is associated with FGFR3 expression level. Bortezomib induced apoptosis in U266 series cells was determined through Annexin V- FITC staining by flow cytometry at 24 hours post treatment at 0.01 μM. *p<0.05, t-test, Y-U266 and T-U266 cells compared with parental cells.*

While there is significant expression seen with all constructs, expression of FGFR3 was the highest with Y-U266 and lowest with K-U266; the latter was less than half the level of Y-U266.

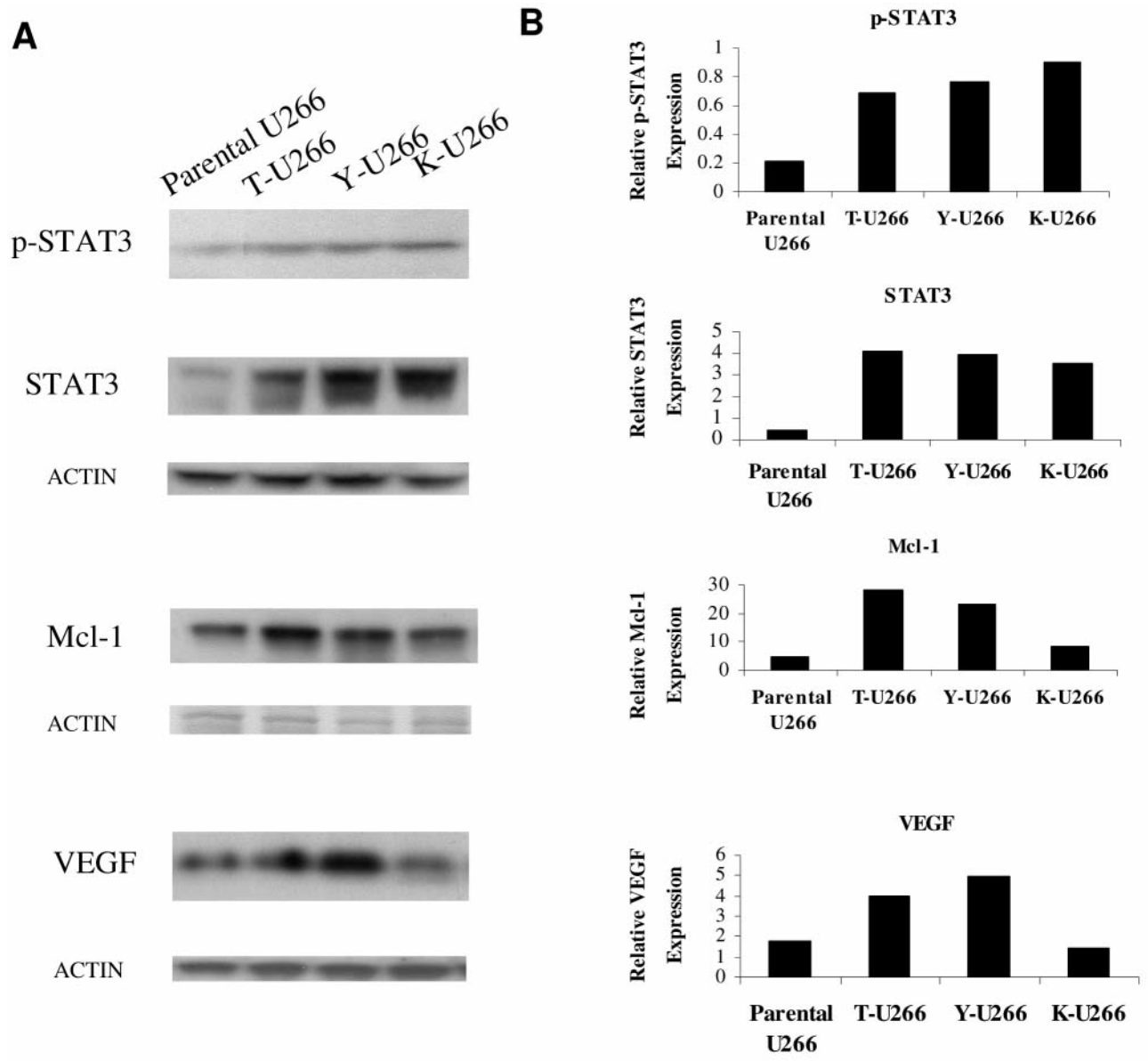

Figure 5. *Increased expression of STAT3, Mcl-1 and VEGF in Y-U266 and T-U266 cells. A, STAT and downstream targets Mcl-1 and VEGF examined by Western blot in transfectants and parental U266 cells. Actin was used as control for protein loading. B, Quantification of proteins by Image Quant 2.5 and corrected by relative controls.*

IC_{50} determination in parental cells and FGFR3 transfectants. To determine the IC_{50} of bortezomib, different transfectants as well as the parental U266 cells were treated with bortezomib at the dose range of 0.0001-10 μM for 24 hours before performing the cell proliferation assay by MTS incorporation. As shown in Figure 2, the cell survival decreased in a dose-dependent manner after treatment with bortezomib in transfected and parental cells. The parental and K-U266 cells survival declined more rapidly after exposure to bortezomib as compared to the Y-U266 and T-U266 cells. Complete cell death was observed at a dose of ≥10 μM in all cell lines. The IC_{50} of bortezomib for Y-U266, T-U266, K-U266 and parental U266 cell lines was approximately 0.08, 0.05, 0.005 and 0.001 μM, respectively, which differ statistically significantly ($p \leq 0.01$).

Y-U266 and T-U266 cells demonstrated bortezomib resistance. To determine whether FGFR3 affects the sensitivity of bortezomib in multiple myeloma, the cell proliferation by MTS incorporation was performed in

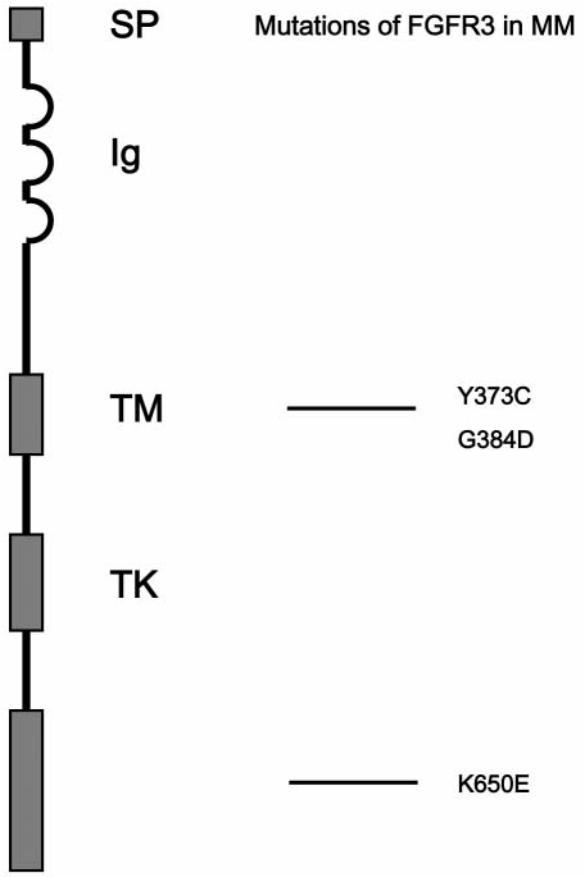

Figure 6. *Mutations of FGFR3 in MM. SP: signal peptide, Ig: immunoglobulin-like domain, TM: transmembrane domain, TK: split tyrosine kinase domain. Modified from Experimental Cell Research 304: 417-431, 2005 (46).*

different transfectants as well as the parental U266 cells. As shown in Figure 3, about 67% of Y-U266 cells and 64% of T-U266 cells survived at 24 hours after exposure to 0.01 μM bortezomib as compared to control cells, whereas only 40% of K-U266 cells and 34% of parental U266 cells survived under similar conditions ($p<0.01$). These results indicate that the parental U266 cells are quite sensitive to bortezomib as compared to the *FGFR3*-transfected U266 cells, which indicates a significant drug-resistant phenotype. Most interestingly, the lower FGFR3 expression clone K-U266 and parental U266 cells show the highest degree of drug sensitivity. On the contrary, Y-U266 and T-U266, which express FGFR3 at a higher concentration, are drug resistant indicating that the effect of bortezomib is associated with FGFR3 expression.

Bortezomib-induced apoptosis is associated with FGFR3 expression level. To determine if apoptotic cell death is induced by bortezomib, the Annexin V–FITC apoptosis assay was performed in FGFR3 transfectants and parental cells. Apoptotic cells were detected by flow cytometry after incubation with 0.01 μM bortezomib for 24 hours. As shown in Figure 4, exposure of Y-U266 and T-U266 cells to bortezomib resulted in only 34% and 32% cells undergoing apoptosis. Moderate apoptosis was observed with K-U266 and parental U266 cells (50% and 60% apoptosis, respectively) ($p\leq0.05$). These findings again suggest that Y-U266 and T-U266 are resistant to bortezomib-mediated apoptosis.

FGFR3 is associated with p-STAT3 signal pathway. To examine the possible signaling pathways mediated by FGFR3, signal pathway factor STAT3 and related downstream targets Mcl-1 and VEGF protein levels were analyzed from cell extracts by Western blot analysis. The quantity for each protein was determined (Image Quant 5.2) and presented in Figure 5, which has been corrected by loading control.

Since STAT3 is a key mediator involved in cytokine signaling of MM cell survival and anti-apoptosis, Western blot analysis was performed using a phospho-specific antibody that binds to phosphorylated STAT3 and total STAT3. As shown in Figure 5, phosphorylation of STAT3 was inhibited in parental U266 cells while phosphorylated STAT3 was significantly enhanced in Y-U266 and T-U266 transfectants. Total STAT3 was elevated in all FGFR3 transfectants. However, K-U266 expressed STAT3 at a slightly lower level than Y-U266 and T-U266. Furthermore, STAT3 downstream factor Mcl-1 was increased accordingly. Expression of Mcl-1 was increased in all transfectants, but weakest in K-U266. Interestingly, the expression of VEGF increased in Y-U266 and T-U266 but decreased in K-U266 and parental U266. These results suggest the drug sensitivity of bortezomib in FGFR3 transfectants may depend on downstream transduction signaling through phosphorylation of STAT3 and VEGF. Other apoptosis and angiogenesis molecules have been examined and no significant changes were seen.

Discussion

In some cases of MM, the translocated *FGFR3* is mutated, mostly in late-stage disease. A recent study of 150 newly diagnosed cases indicates that ~15% of patients have a t(4;14) and 6% of these have a potential activating mutation of the receptor (2, 23, 24). However, it should be noted that *FGFR3* has also been found mutated in a MM tumor not showing the t(4;14) translocation. In that case, it is possible that constitutive activation of FGFR3 would compensate for the absence of overexpression (2, 25, 26). *FGFR3* mutations identified in MM tumors include K650E in OPM2 cells, Y373C in KMS-11 cells, and G384D in KMS-18 cells. The presence of a *FGFR3* mutation appears to be an adverse prognostic factor. It seems that overexpression of wild-type

FGFR3 could be sufficient to lead to MM but its constitutive mutated activation could result in a stronger proliferative signal. To better understand the role of FGFR3 and its mutation in the MM phenotype and development of bortezomib resistance, MM cell line U266, which does not express FGFR3 and absent of t(4;14) translocation, was used in our study. DNA and protein studies confirmed that there is no FGFR3 expression in parental U266 cells, however high levels of FGFR3 are present in the transfected cells, which is comparable to the levels found in MM cells in patients (data not shown).

Cell proliferation as assessed by MTS assay reveals a one log drug resistance in Y373C and wild-type tranfectants as compared to parental U266 cells. Most interestingly, K650E transfectants remain as sensitive to bortezomib as the parental cells. The apoptosis assay results are consistent with the cell proliferation experiments. Apoptosis was less prevalent in Y373C mutant and wild-type *FGFR3*-transfected cells as compared to parental and K650E tranfectants. These results confirm a drug resistance phenotype in Y373C and wild-type transfectant in contrast to a conserved sensitive phenotype in K650E transfectant, which is similar to the parental cells.

Most interestingly, our finding reveals a different response to bortezomib between Y373C and K650E transfectants, which suggests different mutations may affect various pathways of FGFR3 signal transduction. FGFR3 belongs to a family of receptor-tyrosine kinases (PRKs) responding to FGF with four members (FGFR1-4) which share a conserved structure and a high level of amino acid homology (27). Each FGFR is composed of an extracellular ligand-binding domain, a transmembrane domain and a split cytoplasmic tyrosine kinase domain (28). A kinase-activating mutation Y373C and previous reported mutation G384D are identified in the transmembrance domain of FGFR3. Y373C activates FGFR3 by promoting constitutive dimerization in the absence of the ligand (29). The K650E mutation presenting in the germ line is identified in a split cytoplasmic tyrosine kinase domain (Figure 6) (2, 4, 6, 7, 46). K650E mutation is thought to relieve the inhibitory conformation of the kinase enabling its constitutive activation. Y373C signal may be involved in transmemerbrane domain activation, while K650E signal may interact with STATs, ERK and Akt signaling pathway (29, 30). Since the mutations reside within different domains in FGFR3, the downstream signal transduction may vary resulting in the difference of cell phenotype and sensitivity to bortezomib. However, little is known about downstream signaling pathways that are required by FGFR3-induced oncogenesis and cancer cell proliferation. Despite uncertainty about which signaling pathways are involved, the expression of transfected K650E FGFR3 or wild-type FGFR3 plus an FGF ligand in an IL-6-dependent murine plasmacytoma cell line decreases the requirement for IL-6, while enhancing survival and proliferation (3). MAPK, STAT3 and STAT1 phosphorylation in transfected 293T cells with Y373C, K650E and G384D mutated receptors was also observed after starvation (6).

Proteasome inhibitor bortezomib, a boronic acid dipeptide, blocks activation of nuclear factor-kappa B, thereby inhibiting up-regulation of IL-6 induced by multiple myeloma cell-BMSCs binding; it also directly inhibits the growth of myeloma cells and induces their apoptosis by blocking the NF-κB activation. Bortezomib also decreases angiogenic activity in multiple myeloma cells and decreases VEGF secretion and associated angiogenesis (15, 16). Our study indicate bortezomib may be affected and interact with FGFR3 signal transduction in MM. How signaling through FGFR3 can lead to cancer cell proliferation is not fully understood. However, an elevation of STAT3 through ERK can contribute to survival and proliferation of myeloma cell lines has been reported (31). Our findings of increased levels of phosphorylated STAT3 and downstream Mcl-1, which are associated with cell survival and anti-apoptosis, also suggest that FGFR3 signal is mediated through STAT3 in transfected MM cells. Moreover, a recent study by Anderson's group demonstrated down-regulation of MCL-1 contributes to MM cell apoptosis and confers bortezomib resistance (32). Another possible pathway is through VEGF, since increased VEGF expression has been described as a paracrine and autocrine feedback activator (17, 19, 33, 34) and as such, may compromise the effect of bortezomib in transfectant MM cells.

Bortezomib kills multiple myeloma cells; however, prolonged exposure is associated with toxicity and development of bortezomib resistance. To overcome drug resistance, it is essential to examine its mechanism. The contribution of the STAT3 pathway in mediating the environment-medicated drug resistance is under characterization by Dalton's and Jove's group (35). Anderson's group and others have shown that chemoresistance in multiple myeloma cells is conferred by: (a) overexpression of P-glycoprotein; (b) antiapoptotic proteins, such as Bcl-2 or inhibitors of apoptosis proteins; (c) angiogenic factors, such as the direct effects of VEGF on multiple cells (17, 19, 34); (d) defects in drug-induced apoptotic signaling pathways, including those that occur at the level of mitochondria or endoplasmic reticulum; (e) up-regulated expression of growth factor receptors and related signaling pathways; and finally, (f) the interaction between multiple myeloma cells and host bone marrow microenvironment (15, 36). Indeed, it is unlikely that one specific mechanism confers bortezomib resistance and likely that the contribution of diverse factors may lead to the development of drug resistance. *In vitro* studies showed that combining bortezomib with other conventional agents, such as dexamethasone, doxorubicin, melphalan, or mitoxantrone, triggers additive and/or synergistic anti-MM activity (37-41). Taken together our findings indicate

that specific *FGFR3* mutations may allow for more rationale bortezomib combinations, since bortezomib alone may not overcome FGFR3-associated treatment resistance in multiple myeloma (42-45).

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