Inhibition of Bcl-xL by ABT-737 enhances chemotherapy sensitivity in neurofibromatosis type 1-associated malignant peripheral nerve sheath tumor cells

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Abstract. Neurofibromatosis type 1 (NF1) is one of the most commonly inherited autosomal dominant disorders. The malignant peripheral nerve sheath tumor (MPNST) is a major cause of mortality in patients with NF1. In this study, we found that overexpression of Bcl-xL in the established NF1-associated MPNST cell line and primary tissue cultured MPNST cells derived from an NF1 patient was closely associated with anticancer drug resistance of the NF1-associated MPNST cells. We demonstrated that high expression of Bcl-xL in the MPNST cells was caused by a decreased transcriptional expression of the NF1 gene. Downregulation of the NF1 gene by RNA interference (RNAi) induced an increase in Bcl-xL expression and a decrease in its sensitivity to apoptosis in the benign neurofibroma cell line and primary normal cells. These results suggest that an alteration of Bcl-xL expression levels by somatic expression changes in the NF1 locus may contribute to the malignant development of benign tumor tissues or normal tissues to MPNSTs. We further demonstrated that either depletion of Bcl-xL expression by RNAi or inactivation of Bcl-xL by ABT-737, a mimetic of the BH3-only protein BAD, was very effective in sensitizing the MPNST cells to apoptotic cell death by combined treatment with the tested anticancer drug doxorubicin. Notably, a low concentration of ABT-737 and doxorubicin could effectively induce synergistic cytotoxicity in the MPNST cells. These results suggest that pharmacological inhibition of Bcl-xL by ABT-737 in combination with doxorubicin can be a potential therapeutic strategy for the treatment of NF1-associated MPNSTs.

Introduction

Neurofibromatosis type 1 (NF1) is one of the most commonly inherited autosomal dominant human genetic disorder with an incidence of approximately 1 in 3,000-3,500 individuals worldwide (1-3). NF1 has been reported to be caused by de novo mutations in approximately 30-50% of patients (2,3). NF1 is characterized by extremely variable phenotypic features including multiple café-au-lait (CAL) spots, multiple cutaneous neurofibromas, and soft-tissue tumors and has a very poor prognosis (2,3). NF1 is caused by loss-of-function mutations in the NF1 gene encoding neurofibromin, a GTPase-activating protein (GAP) which is one of the major GAP proteins that regulates the small GTPase Ras signaling pathway by converting the active GTP-Ras to an inactive GDP-Ras (4).

Malignant peripheral nerve sheath tumor (MPNST), also referred to as malignant schwannoma or neurofibrosarcoma, is the most frequent malignant neoplasm associated with NF1. MPNST represents a major cause of mortality in patients with NF1 because of its particularly aggressive course (5,6). NF1 is notable for the malignant transformation of benign tumor tissues to MPNSTs. Approximately one half of NF1 patients have benign plexiform neurofibromas (PNs) (2,5). Malignant transformation of benign neurofibromas and PNs to MPNSTs was observed in 2-5% of these patients (2). The lifetime risk for developing MPNSTs in patients with NF1 has been estimated at 8-13% (7).

Although the surgical approach is the mainstay of treatment for NF1, because of very low 5- and 10-year survival rates particularly in male patients with severely malignant NF1-associated MPNSTs characterized by invasive growth, higher propensity to metastasize and limited sensitivity to radiation (7,8), alternative therapeutic approaches have been developed. Recently, there have been several chemotherapy studies on thalidomide (9), interferon α (10), pirfenidone (11), and farnesyltransferase inhibitors (FTI) R11577 (12) for PNs. Phase II clinical trial of pirfenidone in patients with NF1 were
expected to but did not achieve good results (11). A phase I clinical trial of R11577 in patients with NF1-related PNs revealed a limited efficacy of this drug in NF1 (12). In addition, as a clinical therapy for NF1-associated MPNSTs, a combination of chemotherapeutic agents, carboplatin/etoposide (13), cisplatin/adriamycin (14), and ifosfamide/doxorubicin (15,16), have been tested in patients with NF1-associated MPNSTs, after surgical resection and radiation treatments. Long-term investigations using a multimodel therapeutic strategy demonstrated that patients with NF1-associated MPNSTs showed a significantly lower response rate to chemotherapy compared to patients with MPNSTs not associated with NF1 (17), indicating that there are still many hurdles to overcome in chemotherapy for the NF1-associated MPNSTs.

The limited successful results of these clinical studies have led to the discovery of new drugs that mainly target the proteins involved in the Ras-signaling pathway (18). In a recent study, preclinical in vivo evaluation of rapamycin (Sirolimus) or its derivative RAD001 (Everolimus) demonstrated the inhibitory effect of rapamycin on MPNSTs in a xenograft mouse model (19). In addition, B-Raf inhibitor, sorafenib, EGFR inhibitor, erlotinib and R11577/lovastatin mediated the inhibition of cell proliferation in the MPNST cells (20-22).

In this study, we aimed to find new target molecules and/or drugs in order to improve chemotherapy approaches effective in the treatment of the NF1-associated MPNSTs. By comparison analysis between the benign neurofibroma cell line and MPNST cell line and primary normal cells and MPNST cells that bear an identical germ line mutation derived from an NF1 patient, we found overexpression of anti-apoptotic Bcl-xL protein in the MPNST cells, which is responsible for the anticancer drug resistance of the NF1-associated MPNST cells. This finding presented an opportunity to develop new strategies for targeted chemotherapy in the NF1 patients with MPNSTs.

Materials and methods

Drugs. Doxorubicin, cisplatin and etoposide were purchased from Sigma-Aldrich Co. ABT-737 was purchased from Santa Cruz Biotechnology, Inc.

Cell lines. The neurofibromin-deficient MPNST cell line, sNF02.2, and benign the neurofibroma cell line, Hs 53.T, were purchased from the American Type Culture Collection and grown in DMEM media (Hyclone Laboratories) supplemented with 10% FBS (Hyclone Laboratories), penicillin (100 U/ml), and streptomycin (100 µg/ml). All cultured cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Patient and tissue samples. Normal tissue and tumor tissue specimens were obtained by skin biopsy and surgical resection, respectively, from a 24-year-old male patient with NF1 (23,24). The patient having a NF1 nonsense mutation Y2264X (c.6792C>G) in the NF1 gene presented the clinical features of NF1 with CAL spots, scoliosis, cutaneous neurofibromas, subcutaneous neurofibromas, PNs and MPNSTs (23,24). After surgical resection at 24 years of age, the patient died at the age of 25. Three types of tissues, normal phenotypic tissues, PNs and MPNSTs, were pathologically evaluated by routine light microscopy after staining with hematoxylin and eosin (H&E) as previously described (24). The study was approved by the Institutional Review Board Committee of the Ajou University School of Medicine.

Primary tissue culture. Primary tissue culture was performed by primary explant technique. The dissected tissues were finely chopped, rinsed with PBS, and the pieces were seeded onto the surface of a tissue culture flask in 1 ml of DMEM supplemented with a high concentration (40%) of FBS. After an overnight incubation at 37°C, the medium volume was made up to 5 ml and then changed weekly until a substantial outgrowth of cells was observed. Cells were then grown in DMEM media supplemented with 15% FBS. Cells were used from passages 5 through 10. The primary cells from the three types of tissues, normal phenotypic tissues, PNs and MPNSTs, demonstrated their distinct cellular characteristics by western blotting with antibodies against GTP-Ras and its downstream effectors (Fig. 1).

Cell viability assay. The cell viability assay was performed by using the EZ-Cytox Cell Viability Assay kit (Daeil Lab Service, Korea). The cultured cells were plated at a density of 4x10^4 in a 96-well flat-bottom tissue-culture plate, incubated overnight, and were treated with the indicated concentrations of drugs. After 24 h incubation, 10 µl of Ez-Cytox reagent was added, and the cells were incubated for another 2 h and then absorbance was measured at a wavelength of 450 nm with an ELISA microplate reader (Model 680; Bio-Rad).

Gene silencing. The target sequences for the small interfering RNAs (siRNAs) (Genolution, Korea) were as follows: 5'–CAG TGAACGTAAGGGTTTCT-3' for the NF1 gene, 5’-CAGGG
ACAGCATATCAGAG-3' for the BCL2L1 (Bcl-xL) gene and 5'-CTCTACCACCAACTTACGTT-3' for the nonspecific negative control. The siRNAs were diluted in serum-free Opti-MEM (Invitrogen) and transfected into cells using Lipofectamine™ RNAiMax (Invitrogen).

**Results**

**Bcl-xL is overexpressed in the MPNST cells harboring resistance to anticancer drugs inducing apoptosis.** Understanding the mechanism of drug resistance is crucial for developing new strategies for targeted chemotherapy. To examine whether the chemosensitivity to anticancer drugs between the benign neurofibroma and NF1-associated malignant MPNST cells was different, we firstly investigated the cytotoxic sensitivity to the representative anticancer drugs inducing apoptosis in the established cell lines, Hs 53.T and sNF02.2 (25). Since doxorubicin, cisplatin and etoposide have been studied in the patients with NF1-associated MPNSTs (13-16), we used these three anticancer drugs in this study. The cell viability and caspase-3 cleavage assay results showed that the MPNST sNF02.2 cells were more resistant to all three drugs than benign Hs 53.T cells (Fig. 2A-C).

Next, we tried to confirm this result in the NF1-associated primary cells. We performed primary tissue culture of the three types of pathologically evaluated tissues; normal phenotypic tissues (PC-N), benign PNs (PC-B) and malignant MPNSTs (PC-M), derived from a patient with NF1, and demonstrated their distinct cellular characteristics by comparing the levels of GTP-Ras and its downstream effectors, phosphorylated Erk1/2 and phosphorylated Akt, and by the expression level of SOCS3, suppressor of cytokine signaling (Fig. 1). Due to poor proliferation in the primary benign PN cells after 5 passages, the primary normal cells and MPNSTs cells were used in this study. As observed in the cell lines, the primary neurofibromin-deficient MPNST cells were more resistant to all three drugs than the primary neurofibromin-deficient normal phenotypic cells (Fig. 2D-F). In order to understand the reason for the difference in drug resistance between the MPNST cells and benign/normal cells, we further investigated the expression levels of apoptosis-related proteins in these cells. Interestingly, we found that the basal expression level of Bcl-xL was significantly increased in both the MPNST cell line and primary MPNST cells compared to the benign cell line and primary normal cells, while none of the expression levels of other anti-apoptotic proteins Bcl-2 and Mcl-1 were found to be different (Fig. 3A and B) in the MPNST cells compared to that in benign/normal cells.

**Overexpression of Bcl-xL in the MPNST cells is caused by the decreased expression of NF1.** Activated cell survival signaling linked with apoptosis prevention in the NF1-associated MPNSTs is implicated in the activation of the Ras-signaling pathway (4,6). Since the reduced expression of neurofibromin, a negative regulator of this signaling pathway, in the MPNSTs has been reported (26), we firstly investigated the basal expression levels of neurofibromin and observed that the neurofibromin expression levels were significantly lower in both the MPNST cell line and primary MPNST cells compared to the benign/normal cells (Fig. 3A and B). Quantitative RT-PCR revealed that the different expression patterns of neurofibromin and Bcl-xL proteins between the MPNST cells and benign/normal cells originated from the difference in the transcriptional expression of the NF1 and BCL2L1 genes (Fig. 3C and D). To elucidate whether the decreased expression level of neurofibromin in
the MPNST cells was caused by an additional mutation in the intact \(NF1\) allele, we performed molecular analysis of the \(NF1\) gene in the primary MPNST cells. The presence of the normal \(NF1\) allele besides the mutated \(NF1\) allele in trans-chromosomes was confirmed in the primary MPNST cells (data not shown).
Downregulation of neurofibromin expression in the benign neurofibroma cell line/primary normal cells induces an increase in Bcl-xL expression and a decrease in the sensitivity to apoptosis. In order to determine if the expression level of Bcl-xL were dependent on the expression levels of the \( NF1 \) gene, we performed the \( NF1 \) gene silencing experiment in the benign/normal cells. Depletion of neurofibromin expression by siRNA treatment for the \( NF1 \) gene caused an increase in the Bcl-xL expression in a dose-dependent manner in both the benign and normal cells, but it did not have any effect on other anti-apoptotic proteins, Bcl-2 and Mcl-1. Quantitative RT-PCR analysis demonstrated that the knockdown of \( NF1 \) by RNAi led to the increase in the mRNA level of the \( BCL2L1 \) gene (Fig. 4A and C). We next examined whether the depletion of neurofibromin in the benign/normal cells had an influence on the resistance to anticancer drugs. When doxorubicin was co-treated for 24 after 72 h of \( NF1 \) siRNA treatment, the neurofibromin-depleted cells showed increased cell viability and decreased caspase-3 cleavage activity in both the benign and normal cells compared to control RNAi cells (Fig. 4B and D).

Downregulation of Bcl-xL expression by siRNA enhances doxorubicin-induced apoptotic cell death in the MPNST cells. Our results demonstrated that resistance of the MPNST cells to anticancer drugs was caused by increased Bcl-xL expression. Therefore, our study focused on the manipulation of the Bcl-xL expression and the siRNAs targeted against downregulation of Bcl-xL in the MPNST cells. The \( BCL2L1 \) siRNAs per se did not have any effect on cell toxicity in both the MPNST cell line and primary MPNST cells (data not shown). However, co-treatment for 24 h with doxorubicin after 72 h of \( BCL2L1 \) siRNA treatment dramatically reduced the cell viability and increased the caspase-3 cleavage activity in both the MPNST cell line and primary MPNST cells. There was no effect when the negative control siRNAs were co-treated with doxorubicin (Fig. 5). These results indicated that the downregulation of Bcl-xL by RNAi enhanced chemosensitivity of the MPNST cells to the anticancer drug doxorubicin.

ABT-737 in combination with cytotoxic drugs enhances chemotherapy sensitivity in the MPNST cells. We next investigated whether ABT-737 (27), a recently developed Bcl-2 family protein specific inhibitor, had an inhibitory effect against Bcl-xL in the MPNST cells, and whether ABT-737 displayed a synergistic cytotoxicity with anticancer chemotherapeutic agents as in the co-treatment of \( BCL2L1 \) siRNA. The concentration of doxorubicin (0.5-5 µg/ml) required for effective apoptosis was estimated from the results of Figs. 2 and 5. ABT-737 alone did not have an effect on cell toxicity in both the MPNST cell line...
and primary MPNST cells at the concentrations tested (1-80 µM) (data not shown). However, when the cells were co-treated with doxorubicin, ABT-737 effectively enhanced apoptotic cell death in a dose-dependent manner in both the MPNST cell line and primary MPNST cells, compared to cells with single treatment of doxorubicin (Fig. 6). Notably, ABT-737 in combination with doxorubicin dramatically enhanced chemotherapy sensitivity in the MPNST cells. Furthermore, ABT-737 effectively reduced the dosage of doxorubicin required for efficacious MPNST cell death. The concentrations of both ABT-737 and doxorubicin required for causing approximately 80% cytotoxicity in the established and primary tissue cultured NF1-associated MPNST cells were calculated as; 1 µM of ABT-737 plus 2.5-5 µg/ml of doxorubicin.

Discussion

Since haploinsufficiency of neurofibromin activity by NF1 mutation exists in all the cells of the NF1 patient, even in the normally functional cells, it has been suggested that the additional genetic or epigenetic changes may participate in malignant development of benign tumor tissues to MPNSTs as well as in tumorigenesis of NF1 (4). Somatic loss of heterozygosity (LOH) at the NF1 locus and genomic imbalances in chromosomes 17, 19 and 22q are responsible for this tumor development (28,29). Furthermore, mutations and/or gene expression changes in many genes such as CD44, CDKN2A, EGFR, PTEN, RB1, SOX9 and TP53, have also been reported (4,5). Recently, genome-wide transcriptome analyses revealed that p53 inactivation mediated loss of miR-34a expression in MPNSTs (30). However, the exact molecular pathogenesis of malignant transformation of benign tumor tissues to MPNSTs in NF1 patients has not yet been elucidated.

In this study, we demonstrated that Bcl-xL was over-expressed in the established and primary tissue cultured NF1-associated MPNST cells compared to the benign neurofibroma cell line and primary normal cells. This originated from the decreased transcriptional expression of the NF1 gene, despite no additional mutations in the normal NF1 allele besides the mutant NF1 allele. The reduced expression of neurofibromin, a negative-regulator for the Ras-signaling pathway,
in the MPNSTs has been reported (26). However, no frequent somatic mutations in the NF1 gene or hypermethylation of the NF1 gene have been detected in MPNSTs (31). We also investigated the methylation levels in the promoter region of the NF1 gene in the primary MPNST and normal cells by DNA methylation chip analysis using the GoldenGate Methylation Cancer Panel I (Illumina) but did not detect hypermethylation in both the cell types (data not shown). Overexpression of the BCL2L1 mRNA in MPNSTs can be explained by the decreased NF1/neurofibromin expression. Since Ets and NF-kB, the downstream proteins in the Ras-signaling pathway, are well known as the main transcriptional factors for the BCL2L1 gene (32), deficiency in neurofibromin can cause hyperactivation of Ras-signaling due to the promotion of Ets and NF-kB expression. These results suggest that alteration in Bcl-xL expression level is caused by somatic expression changes in the intact NF1 locus and not by somatic NF1 mutation. The role of Ras-signaling pathway has been closely implicated in malignant transformation and drug resistance in many types of cancers (18). Although the molecular mechanisms for somatic loss of NF1 in the MPNST cells have not been elucidated, loss of neurofibromin may directly contribute to Bcl-xL overexpression through the activation of the pathway and may further contribute to malignant development of benign tumor tissues or normal tissues to MPNSTs.

Chemoresistance in NF1-associated MPNSTs has been poorly discussed so far. Our results have demonstrated that overexpression of Bcl-xL is a principal cause for drug resistance of the MPNST cells to anticancer drugs. Apoptotic Bcl-2 family proteins, Bcl-2, Bcl-xL, McI-1 and Bcl-w, which contribute to tumorigenesis, tumor progression and tumor chemoresistance are known to be overexpressed in many cancers (33). Particularly, Bcl-xL overexpression is involved in resistance to a large number of cytotoxic agents in human cancer cell lines (34). Therefore, Bcl-xL is considered one of the promising targets for overcoming drug resistance by enhancing apoptosis in malignant tumor cells. Inhibition of Bcl-xL by antisense oligonucleotides or siRNAs significantly enhanced chemosensitivity to cisplatin (35,36). In addition, various non-peptidic small molecule inhibitors against Bcl-2 family proteins have been developed and preclinical or clinical trials for various cancer therapies have been performed (37). Among them, ABT-737, a mimetic of the BH3-only protein BAD, and its modified form ABT-263 are drawing attention as good candidates to selectively target cancer cells and are in the phase I/II of clinical trials for various cancer therapies (38). ABT-737 selectively inhibits Bcl-2, Bcl-xL, and Bcl-w and synergizes with conventional chemotherapeutic drugs to promote apoptosis in multiple cancer types (38,39). Our results showed that either depletion of Bcl-xL expression by RNAi or inactivation of Bcl-xL by ABT-737 enhanced doxorubicin-induced apoptosis in MPNST cells (Figs. 5 and 6). However, regarding the possibilities and limitations of its clinical application, ABT-737 is considered more effective than BCL2L1 siRNA. Single treatment with ABT-737 did not exert a cytotoxic effect in the MPNST cells as previously reported in other types of cells (38), but, a low concentration (1 μM) of ABT-737 could significantly enhance the cytotoxic effect of doxorubicin, when used as combination therapy in our study. Previously, many cancer cell types were shown to be refractory to ABT-737 because of high expression of Mcl-1 (40,41). However, our MPNST cells did not exhibit higher Mcl-1 expression levels compared to the benign and normal cells (Fig. 2), thereby suggesting a beneficial effect of ABT-737 in NF1-associated MPNSTs. Doxorubicin is a clinically used anticancer drug that functions as topoisomerase II inhibitor and forms covalent DNA adducts. In fact, doxorubicin recently studied as a chemotherapeutic agent in combination with ifosfamide in patients with NF1-associated MPNSTs (15,16) and in combination with ABT-737 in promyelocytic leukemia cells and chondrosarcoma cell (42,43). Our results suggest that the combination of ABT-737 and doxorubicin is very effective in enhancing chemotherapy sensitivity in the NF1-associated MPNST cells.

In conclusion, to the best of our knowledge, this is the first study to demonstrate that overexpression of Bcl-xL caused by downregulation of NF1 is closely associated with drug resistance in the NF1-associated MPNST cells and suggests that Bcl-xL inhibition by ABT-737 in combination with doxorubicin can be a potential therapeutic strategy for the treatment of the NF1-associated MPNSTs. Further studies are necessary to evaluate this combination therapy in preclinical models of MPNSTs. We believe that this study will have a significant impact in the field of cancer and will be helpful to those studying chemotherapy and molecular mechanisms involved in the pathogenesis of NF1-associated MPNSTs.

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References


