NF1 deficiency causes Bcl-xL upregulation in Schwann cells derived from neurofibromatosis type 1-associated malignant peripheral nerve sheath tumors

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Abstract. Since the bi-allelic inactivation of both neurofibromin 1 (NF1) gene alleles (NF1−/−) in Schwann cells (SCs) is common in both benign plexiform neurofibromas (PNs) and malignant peripheral nerve sheath tumors (MPNSTs) in patients with neurofibromatosis type 1 (NF1), other genetic alterations in SCs may be required for tumor progression of PNs to MPNSTs. We found that the anti-apoptotic Bcl-xL protein is upregulated in MPNST tissues compared to PN tissues from patients with NF1 by immunohistological staining. In addition, we investigated whether Bcl-xL is upregulated in SCs derived from MPNSTs and found a significantly higher Bcl-xL expression level in sNF96.2 MPNST-derived SCs compared to normal human SCs (HSCs). We also discovered that the increased Bcl-xL expression caused an increase in drug resistance to doxorubicin in MPNST-derived SCs. Manipulation of NF1 gene expression levels by treatment with small interfering RNA (siRNA) and overexpression of the neurofibromin GAP-related domain (NF1-GRD) demonstrated that upregulation Bcl-xL expression in MPNST-derived SCs was caused by NF1 deficiency. Treatment with the Erk1/2 inhibitor, PD98059, resulted in a slight increase in Bcl-xL levels in neurofibromin-depleted normal HSCs, indicating that Bcl-xL upregulation in MPNST-derived SCs is mediated by activated Erk1/2, which is a Ras downstream protein regulated by neurofibromin. As the reduction of Bcl-xL expression restored sensitivity to doxorubicin-induced apoptosis in sNF96.2 cells, we examined the effect of the small molecule Bcl-xL inhibitor ABT-737 on sNF96.2 cells. A very low dose of ABT-737 combined with doxorubicin synergistically enhanced sensitivity to doxorubicin-induced apoptosis in sNF96.2 cells, suggesting that ABT-737 and doxorubicin may be a good combination to effectively treat NF1-associated MPNSTs with minimal side-effects. Collectively, our results suggest that upregulation of Bcl-xL in MPNST-derived SCs may be caused by the NF1 deficiency-mediated elevation in Ras/MAPK signaling and may provide a new potential chemotherapeutic target in patients with NF1 and MPNSTs.

Introduction

Neurofibromatosis type 1 (NF1; MIM #162200) is a commonly inherited autosomal dominant disorder characterized by variable phenotypic features, including cutaneous manifestations, such as café au lait spots, neurofibromas and freckling of the axillary or inguinal regions, as well as extracutaneous manifestations such as Lisch nodules, optic nerve gliomas, scoliosis, bone dysplasia, malignant tumors, and cognitive impairment (1,2). NF1 is caused by neurofibromin 1 (NF1) gene mutations, which encode neurofibromin, a GTPase-activating protein (GAP) (3). The majority of patients with NF1 develop benign dermal neurofibromas (DNs) and/or plexiform neurofibromas (PNs) (4,5).

Neurofibromas are composed of a mixture of cell types including Schwann cells (SCs), fibroblast cells, mast cells, and perineural cells (6). SCs are believed to be the primary pathogenic cell source in neurofibromas (7). As the complete loss of the NF1 gene has been identified exclusively in neurofibroma SCs (8-10) and the loss of NF1 in the SC lineage is sufficient to generate tumors in mice (11), the bi-allelic inactivation of both NF1 alleles (NF1−/−) in SCs by germline NF1 mutation at one allele and the additional somatic loss of heterozygosity (LOH) at the remaining functional NF1 locus, has been suggested to be a major cause of NF1 tumorigenesis. In addition, haploinsufficiency in other types of cells (NF1−/+ in neural crest-derived tissues, including fibroblast cells, mast cells and perineural cells also plays an important role in the pathobiology of NF1 (6,12,13).
Malignant peripheral nerve sheath tumors (MPNSTs) are a type of aggressive sarcoma and are a major cause of mortality in patients with NF1 (5,14-16). The lifetime risk of developing MPNSTs in patients with NF1 is 8-13% (17) or 5.9-10.3% (18). The majority of NF1-associated MPNSTs (approximately 85% of cases) are high-grade malignant tumors. The malignant transformation of benign PNs to MPNSTs in patients with NF1 is notable (19) and is of far greater concern to patients with NF1 (20); however, the pathogenesis is poorly understood. The bi-allelic inactivation of the NF1 gene caused by a germline first-hit mutation and a somatic second-hit LOH in SCs has been identified in DNs (21,22), PNs (23,24), and MPNSTs (24,25) in patients with NF1, indicating that the complete loss of the NF1 gene (NF1−/−) in SCs contributes to benign neurofibroma formation and progression to MPNSTs. Since bi-allelic inactivation of the NF1 gene is insufficient to explain the pathogenesis of tumor progression in NF1, cooperating genetic or epigenetic changes have been suggested to be involved in MPNST pathogenesis. Hence, robust histological and molecular analyses have been conducted to compare neurofibromas and MPNSTs (15,26,27), and recently developed genome-wide DNA copy number change profiling using array comparative genomic hybridization has identified causal genes in MPNST development (28,29). To date, genes involved in regulating the cell cycle and growth signal transduction have been reported mainly to be dysregulated in MPNSTs (6,30,31).

Since bi-allelic inactivation of the NF1 gene is insufficient to explain the pathogenesis of tumor progression in NF1, cooperating genetic or epigenetic changes have been suggested to be involved in MPNST pathogenesis. Hence, robust histological and molecular analyses have been conducted to compare neurofibromas and MPNSTs (15,26,27), and recently developed genome-wide DNA copy number change profiling using array comparative genomic hybridization has identified causal genes in MPNST development (28,29). To date, genes involved in regulating the cell cycle and growth signal transduction have been reported mainly to be dysregulated in MPNSTs (6,30,31).

A number of studies have focused on genetic alterations in SCs, as most MPNSTs are thought to arise from SCs (7,32).

We unexpectedly found that the anti-apoptotic protein, Bcl-xL, is upregulated in primary-cultures and established NF1-associated MPNST cells. Bcl-xL is responsible for the acquired anticancer drug resistance of MPNST cells (33). In this study, we compared Bcl-xL expression levels between normal and MPNST-derived SCs, as well as between PNs and MPNST tissues from patients with NF1 to determine whether Bcl-xL upregulation in SCs is involved in MPNST pathogenesis. Furthermore, we also examined changes in Bcl-xL expression levels and sensitivity to apoptosis induced by anticancer drugs in NF1−/− and NF1−/− SCs when NF1 expression was manipulated to determine whether Bcl-xL upregulation is associated with NF1 deficiency in SCs.

Materials and methods

Antibodies and reagents. Anti-Bcl-xL, anti-Bcl2, anti-Bax, anti-caspase 3, anti-extracellular signal-regulated kinase (Erk)1/2 and anti-phosphorylated Erk1/2 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-neurofibromin, anti-α-tubulin, anti-p53, anti-Mcl-1, HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-S100 and anti-GFP antibodies were purchased from Thermofisher Scientific (Rockford, IL, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Doxorubicin and PD98059 were obtained from Sigma-Aldrich (St. Louis, MO, USA). ABT-737 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the American Type Culture Collection (Manassas, VA, USA), respectively. Cells were cultured in Dulbecco's modified Eagle medium (HyClone Laboratories, Logan, UT, USA) containing 10% fetal bovine serum supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C under a humidified atmosphere containing 5% CO2.

Hematoxylin and eosin (H&E) staining. Tumor tissues were obtained from patients with NF1 by surgical resection. The specimens were formalin-fixed and embedded in paraffin wax for pathological evaluation by routine light microscopy. Serial 3-µm sections were prepared on glass using a cryostat, and the slides were stained with H&E.

Immunohistochemistry (IHC). Formalin-fixed paraffin-embedded (FFPE) blocks from six patients with NF1 were cut at 10 µm, and the sections were dewaxed, rehydrated, followed by antigen retrieval in boiling citrate buffer. Immunostaining was carried out using an Ultravision LP-HRP Polymer DAB kit (Thermo Fisher Scientific, Kalamazoo, MI, USA), according to the manufacturer's instructions. Briefly, the sections were incubated with Ultra V Block (Lab Vision, Kalamazoo, MI, USA) for 5 min at room temperature to reduce the non-specific background, and were then treated with hydrogen peroxide to block endogenous peroxidase activity. The sections were incubated with primary antibody for 1 h and then incubated with HRP polymer for 20 min. The reaction product was visualized with DAB chromogen. Pathological evaluation was performed under light microscopy. The present study using human FFPE samples was approved by the Institutional Review Board of the Ajou University School of Medicine, Suwon, Korea.

Plasmid constructs and small interfering RNAs (siRNAs). Plasmid constructs encoding wild-type Bcl-xL were generated as described previously (34). The cDNA of the GAP-related domain (GRD) region (1,181 bp) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using the primers to generate the plasmid construct expressing the human NF1-GRD: 5′-ATAGATCTACCATGGATCTCCAGA CAAAGAGCTACATTTATG-3′ and 5′-GTAAGCTTAACCAG TTGTTGTATCTGCCACAGGT-3′, from total RNAs of human NF1-GRD: 5′-ATAGATCTACCATGGATCTCCAGA CAAAGAGCTACATTTATG-3′ and 5′-GTAAGCTTAACCAG TTGTTGTATCTGCCACAGGT-3′, from total RNAs of human skin tissue cultured fibroblast cells. The cDNAs were subcloned into the pEYFP-C1 vector (Clontech, Palo Alto, CA, USA) using the BglII and HindIII restriction enzyme sites. The siRNAs were synthesized by Genolution Pharmaceuticals, Inc. (Seoul, South Korea). The target sequences for the siRNAs were as follows: 5′-CATGTAACGTAAAGGTTCTC-3′ for the NF1 gene, 5′-CAGGGACAGCATATCGAG-3′ for the BCL2L1 (Bcl-xL) gene and 5′-CCTACGCGACATCTTGT-3′ for the non-specific negative control. Cell transfection of the siRNAs and plasmid constructs was conducted using Lipofectamine RNAiMAX (Invitrogen) and Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer's instructions.

Cell viability assay. Cell viability was assessed using the EZ-Cytox Cell Viability Assay kit (Daeil Labservice, Seoul, Korea). Cells were seeded in a 96-well tissue-culture plate (7x103 cells/well), cultured overnight, and then treated with various concentrations of doxorubicin and/or ABT-737. After 24 h of
incubation, 10 µl of Ez-Cytox reagent was added to each well, and the cells were incubated for a further 2 h. The plate was read with an enzyme-linked immunosorbent assay microplate reader (Bio-Rad Model 680; Hercules, CA, USA) at 450 nm.

**Real-time RT-PCR.** Total RNAs were isolated from the cultured cells using TRIzol reagent (Invitrogen), treated with RNase-free DNase I (Invitrogen) to avoid amplification of genomic DNA, and were subsequently reverse-transcribed using the RevertAid™ H Minus First-Strand cDNA Synthesis kit (Fermentas, Burlington, ON, Canada) with the oligo(dT)15-18 primer. Real-time RT-PCR was performed using the SYBR-Green I qPCR kit (Takara, Shiga, Japan). The specific primers used were as follows: 5’-GTGGATCGCAGCTGGATGGCCAC-3’ and 5’-CTGACCAAGGCAGCTGGAGCTTGAAGCT-3’ for *BCL2L1*. The P23828 primer set (Bioneer, Seoul, Korea) was used for *NF1* and 5’-TGATTCCACAGTACTCCGCTCCACGGAACCAGCGCTTGAAGCTTGAAGCT-3’ and 5’-CTGACCAAGGCAGCTGGAGCTTGAAGCT-3’ for the *GAPDH* gene (a relative quantification standard). All real-time RT-PCR measurements were performed using the ABI Prism 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA).

**Western blot analysis.** Cultured cells were lysed in RIPA buffer [150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris buffer, pH 8.0]. Proteins were heated at 100°C for 10 min and analyzed by SDS-polyacrylamide gel electrophoresis on 8-12% polyacrylamide gels. The proteins were electroblotted onto PVDF membranes (Millipore, Milford, MA, USA). The membrane blots were blocked with 5% (w/v) non-fat dried milk, incubated with primary and secondary antibodies, and then visualized with the Enhanced Chemiluminescence Western Blotting Detection System (WEST-ZOL plus; iNtRON Biotechnology, Daejeon, Korea).

**Statistical analysis.** In this study, the results are expressed as the means ± standard deviation. All experiments were repeated at least three times. Statistical significance was determined by the two-tailed Student’s t-test, and *P*-values <0.05 were considered to indicate statistically significant differences.

**Results**

**Higher Bcl-xL expression observed in MPNSTs compared to PNs in patients with NF1.** Since Bcl-xL hyperexpression has been observed in NF1-associated MPNST cells (33), we confirmed this finding in the tumor tissues of patients with NF1. Tumor specimens were obtained by surgical resection from six patients with NF1. The patients were diagnosed with NF1 at the Ajou University Hospital according to NF1 diagnostic criteria (35). The clinical features of the patients are summarized in Table I. Histopathological analysis of the tumor specimens by H&E staining revealed that three patients [patient (P1)-P3] had benign PNs and three patients (P4-P6) had MPNSTs (Table I). The H&E results of P1 and P4 are shown in Fig. 1. The PNs were composed of loosely spaced tumor cells in a myxoid matrix or collagenous strands, whereas MPNSTs showed densely cellular atypical spindle cells forming intersecting fascicles. We then compared the Bcl-xL

<table>
<thead>
<tr>
<th>ID</th>
<th>Gender</th>
<th>Age at diagnosis</th>
<th>H&amp;E findings</th>
<th>Bcl-xL</th>
<th>S100</th>
<th>NF1 gene mutation</th>
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<td>P1</td>
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<td>59</td>
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<td>+</td>
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<td>Benign</td>
<td>++</td>
<td>++</td>
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<td>Malignant</td>
<td>+</td>
<td>+</td>
<td>c.4861_4862GT&gt;AG</td>
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<td>+</td>
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<td>41</td>
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<td>+</td>
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ID, identity; H&E, hematoxylin and eosin staining; N/A, not applicable. +, expressed; ++, overexpressed; Y, yes; N, no.
expression levels between the PN and MPNST tumor tissues
by IHC analysis. The IHC evaluation revealed a higher Bcl-xL
expression in MPNSTs (P4-P6) compared to PNs (P1-P3) in
all patients with NF1 tested.

SCs are generally thought to be the major progenitors of
neurofibromas and MPNSTs and characteristically express the
S100 protein (36). IHC staining using an antibody against the
SC lineage marker, S100, showed that all tumors contained
S100-positive cells (Table I) and more S100-positive cells
were present in MPNSTs than in PNs, as shown in P1 and
P4 (Fig. 1). These results suggest that tumors from patients
with NF1 mainly originate from SCs and that most Bcl-xL-
overexpressing cells are SC lineage cells.

**Upregulation of Bcl-xL causes an increase in resistance to
doxorubicin in NF1-deficient MPNST SCs.** We examined
whether the basal Bcl-xL expression levels were different
between SCs derived from normal tissues and those derived
from MPNSTs. We compared the normal human SC line
(HSC) and the sNF96.2 SC line, which was derived from a
MPNST in a patient with NF1. HSCs have both normal
NF1 alleles (NF1+/+), whereas the sNF96.2 cells have a complete
LOH and no remaining NF1 allele (NF1−/−) (37). Western blot
analysis for neurofibromin confirmed normal neurofibromin
expression in the HSCs and null neurofibromin expression in
the sNF96.2 cells (Fig. 2A). The increased pErk1/2 protein
level involved in the Ras/Raf/Mek/Erk signaling pathway (38),
demonstrated that the sNF96.2 cells were MPNST-derived SCs
(Fig. 2A). Basal Bcl-xL expression was significantly upregu-
lated in the sNF96.2 cells compared to the HSCs, whereas the
lower expression of Bcl-2, another anti-apoptotic protein, was
observed in the sNF96.2 cells (Fig. 2A). No changes in Mcl-1
and p53 expression levels were observed between the two cell
lines.

Chemoresistance in NF1-associated MPNST cells has
been recently reported (33). We examined sensitivity to
apoptosis induced by anticancer drugs in HSCs and sNF96.2
cells to determine whether SCs are responsible for chemoresistance in MPNSTs. We used doxorubicin as doxorubicin has
been suggested to be a good candidate for MPNST chemo-
therapy (33,39). The cell viability assay results demonstrated
that the sNF96.2 cells were more resistant to doxorubicin than
the HSCs (Fig. 2B), suggesting that the upregulation of Bcl-xL
may decrease apoptosis sensitivity in sNF96.2 cells.

**Bcl-xL expression level is closely related to sensitivity to doxo-
rubicin-induced apoptosis in normal SCs and NF1-deficient
MPNST-derived SCs.** We manipulated Bcl-xL expression levels
in HSCs and sNF96.2 cells to determine whether the sensitivity
to doxorubicin-induced apoptosis in SCs was dependent on
Bcl-xL expression level. The overexpression of Bcl-xL in HSCs
decreased caspase 3 cleavage activity significantly and increased
cell viability following doxorubicin treatment (Fig. 3A and B).

By contrast, the downregulation of Bcl-xL in the sNF96.2 cells
following treatment with siRNAs targeting the BCL2L1 gene
significantly increased caspase 3 cleavage activity and reduced
cell viability following doxorubicin treatment (Fig. 3C and D).

These results indicate that the Bcl-xL level is closely related to
sensitivity to doxorubicin-induced apoptosis in both types of
SC (HSCs and MPNST-derived SCs).

**Bcl-xL expression level is mediated by NF1 gene level in
normal SCs and NF1-deficient MPNST-derived SCs.** The
genetically noticeable difference between HSCs and sNF96.2
cells is determined by whether the NF1 gene is intact (NF1+/+)
or inactivated (NF1−/−), suggesting that the hyperexpression of
Bcl-xL in sNF96.2 cells may be mediated by decreased NF1
expression. We manipulated neurofibromin expression levels
in HSCs and sNF96.2 cells to determine whether Bcl-xL
expression is dependent on neurofibromin expression in SCs.
The downregulation of neurofibromin expression by siRNA
targeting the NF1 gene in HSCs caused an increase in Bcl-xL
expression and pErk1/2 protein levels, but had no effect on
other apoptosis-related proteins such as Bcl-2, Mcl-1, Bax and
p53 (Fig. 4A). Real-time PCR results of BCL2L1 demonstrated
that the increased Bcl-xL expression level in the neurofibromin-
depleted HSCs was caused by the increased BCL2L1 mRNA
expression (Fig. 4B). The neurofibromin-depleted HSCs

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**Figure 1. Immunohistochemical staining (IHC) of Bcl-xL in the plexiform neurofibroma (PN) and malignant peripheral nerve sheet tumor (MPNST) tissues from patients with neurofibromatosis type 1 (NF1). Histological analysis by standard hematoxylin and eosin (H&E) staining and IHC using antibodies against S100 and Bcl-xL were carried out on PN and MPNST tissue sections.**

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<tr>
<th>H&amp;E</th>
<th>IHC: S100</th>
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Figure 2. Comparison of a normal human Schwann cell line (HSC) and human NF1-depleted Schwann cell line characteristics (sNF96.2). (A) Endogenous protein levels of extracellular signal-regulated kinase (Erk1/2), phosphorylated Erk1/2 (pErk1/2), Bcl-xL, Bcl-2, Mcl-1, Bax, p53 and α-tubulin in the HSC and sNF96.2 cells were assessed by western blot analysis. The α-tubulin protein level was used as the internal control. (B) Sensitivities to apoptosis induced by the anticancer drug, doxorubicin, in HSCs and sNF96.2 cells were measured by cell viability assay. The HSCs and sNF96.2 cells were treated with the indicated concentrations of doxorubicin, and cell viability was determined by the Ez-Cytox assay after 24 h of incubation.

Figure 3. Bcl-xL level-dependent alteration of sensitivity to doxorubicin-induced apoptosis in the normal human Schwann cell line (HSC) and human NF1-depleted Schwann cell line (sNF96.2). (A and B) Overexpression of Bcl-xL in HSCs. The HSCs were transfected with pcDNA3.1 plasmid vector or a Bcl-xL plasmid construct and then incubated for 24 h. The cells were further treated with 0.5 µg/ml doxorubicin for 24 h. (C and D) Knockdown of Bcl-xL using small interfering RNAs (siRNAs) in sNF96.2 cells. sNF96.2 cells were treated with BCL2L1 gene siRNAs (50 nM) or the non-specific negative control (50 nM) and incubated for 72 h. The cells were further treated with 0.5 µg/ml doxorubicin for 24 h. (A and C) Protein levels of Bcl-xL, uncleaved caspase 3 (U-caspase 3), cleaved caspase 3 (C-caspase 3), and α-tubulin were determined by western blot analysis. The α-tubulin protein level was used as the internal control. (B and D) Cell viabilities of the untreated (None) and doxorubicin treated (Doxorubicin) cells that were transfected with control or BCL2L1 siRNAs were determined by the Ez-Cytox assay. *P<0.05 and **P<0.01.
showed decreased caspase 3 cleavage activity and increased cell viability following doxorubicin treatment for 24 h after 72 h of NF1 siRNA treatment (Fig. 4C and D), similar to the Bcl-xL-overexpressing HSCs (Fig. 3A and B).

We then overexpressed NF1 in sNF96.2 cells. Since the NF1 gene is very large, and the NF1-GRD is sufficient to restore normal growth in mouse NF1-/- cells (40), we constructed a human NF1-GRD fused to GFP. NF1-GRD-GFP overexpression in the sNF96.2 cells significantly increased caspase 3 cleavage activity and reduced cell viability following doxorubicin treatment (Fig. 5A and B), similar to the Bcl-xL-depleted sNF96.2 cells (Fig. 3C and D). These results indicate that Bcl-xL expression level is mediated by the NF1 gene level in both types of SC (HSCs and MPNST-derived SCs).

**NF1 deficiency induces Bcl-xL expression by activating Erk1/2 in the Ras/mitogen-activated protein kinase (MAPK) signaling pathway.** We then wished to clarify the molecular mechanisms by which alterations in neurofibromin expression in SCs modulate the Bcl-xL expression level. As shown in Fig. 2A, Erk1/2 was highly activated in the sNF96.2 cells when Bcl-xL was highly expressed, suggesting that the activation of the Erk1/2 downstream effector in the Ras/MAPK signaling pathway may be involved in NF1 deficiency-mediated Bcl-xL upregulation in MPNST-derived SCs. We first examined whether the inhibition of Erk1/2 could influence the Bcl-xL expression level in sNF96.2 cells. When the sNF96.2 cells were treated with the Erk1/2 inhibitor, PD98059, for 24 h, the Bcl-xL protein level decreased in a dose-dependent manner (Fig. 6A). Subsequently, the neurofibromin-depleted HSCs following transfection with NF1 siRNAs exhibited a significant increase in pErk1/2 and Bcl-xL levels in the absence of PD98059; however, the neurofibromin-depleted HSCs showed a slight increase in pErk1/2 and Bcl-xL levels in the presence of PD98059. We then wished to clarify the molecular mechanisms by which alterations in neurofibromin expression in SCs modulate the Bcl-xL expression level. As shown in Fig. 2A, Erk1/2 was highly activated in the sNF96.2 cells when Bcl-xL was highly expressed, suggesting that the activation of the Erk1/2 downstream effector in the Ras/MAPK signaling pathway may be involved in NF1 deficiency-mediated Bcl-xL upregulation in MPNST-derived SCs. We first examined whether the inhibition of Erk1/2 could influence the Bcl-xL expression level in sNF96.2 cells. When the sNF96.2 cells were treated with the Erk1/2 inhibitor, PD98059, for 24 h, the Bcl-xL protein level decreased in a dose-dependent manner (Fig. 6A). Subsequently, the neurofibromin-depleted HSCs following transfection with NF1 siRNAs exhibited a significant increase in pErk1/2 and Bcl-xL levels in the absence of PD98059; however, the neurofibromin-depleted HSCs showed a slight increase in pErk1/2 and Bcl-xL levels in the presence of PD98059.

Figure 4. An increase in Bcl-xL expression and a decrease in sensitivity to doxorubicin-induced apoptosis in the normal human Schwann cell line (HSC) by NF1 knockdown. (A) The HSCs were transfected with NF1 gene siRNAs (100 nM) or the non-specific negative control (100 nM) and then incubated for 72 h. Protein levels of extracellular signal-regulated kinase (Erk)1/2, phosphorylated Erk1/2 (pErk1/2), Bcl-xL, Bcl-2, Mcl-1, Bax, p53 and α-tubulin in the HSCs were assessed by western blot analysis. The α-tubulin protein level was used as the internal control. (B) Relative NF1 and BCL2L1 mRNA levels in HSCs transfected with control or NF1 siRNAs and assessed by real-time reverse transcription-polymerase chain reaction (RT-PCR). **P<0.01. (C and D) The HSCs transfected with control or NF1 siRNAs were further treated with 0.5 µg/ml doxorubicin for 24 h. Protein levels of neurofibromin, uncleaved caspase 3 (U-caspase 3), cleaved caspase 3 (C-caspase 3), and α-tubulin were determined by western blot analysis. The α-tubulin protein level was used as the internal control. (D) Cell viabilities of the untreated (None) and doxorubicin treated (Doxorubicin) cells transfected with control or NF1 siRNAs were determined by the Ez-Cytox assay. *P<0.05.
of PD98059 (Fig. 6B). These results suggest that the Erk1/2 activation level may play a crucial role in the NF1 dose-dependent Bcl-xL expression changes in both SCs.

The Bcl-xL inhibitor, ABT-737, synergistically enhances sensitivity to doxorubicin-induced apoptosis in NF1-deficient MPNST-derived SCs. Chemotherapy for NF1-associated MPNSTs has not been extensively investigated. ABT-737, a mimic of the BH3-only protein Bad and which binds selectively to Bcl-2, Bcl-xL and Bcl-w (41), induces synergistic cytotoxicity in MPNST cells when combined with doxorubicin (33). We then investigated whether ABT-737 would exert a synergistic cytotoxic effect in sNF96.2, Bcl-xL and α-tubulin in the SCs assessed by western blot analysis. The α-tubulin protein level was used as the internal control.

Discussion

Mouse model studies have reported that the double-hit inactivation of the NF1 gene (NF1−) in SCs leads benign DNs and/or PNs to form tumors (42). Other genetic alterations in SCs may be required for tumors to progress from PNs to MPNSTs. As loss-of-function mutations in tumor suppressor genes, such as TP53, RB1 and CDKN2A are particularly common in MPNSTs (31), the accumulation of additional loss-of-function mutations in these tumor suppressor genes in NF1− SCs may be required for MPNST pathogenesis. In addition, the dysregulation of many
genes in MPNSTs has been reported (7,31). In particular, a large-scale comparison between human MPNST-derived SCs and normal SCs revealed a relative downregulation of the SC differentiation markers, SOX10, CNP and PMP22, and nerve growth factor receptor, as well as the relative upregulation of the neural crest stem cell markers, SOX9 and TWIST1, in MPNST-derived SCs (27). Post-transcriptional modification by microRNAs has also been studied in NF1 and the results showed the upregulation of miR-10b (43) and the downregulation of miR34a whose expression is mediated by p53 (44) in MPNST-derived SCs or tissues.

We recently reported the hyperexpression of the anti-apoptotic protein, Bcl-xL, in primary MPNST cells and a MPNST cell line (33). Hence, in this study, we aimed to confirm this result in patient samples as a first step and demonstrated the upregulation of Bcl-xL in MPNSTs from patients with NF1 by Bcl-xL immunohistological staining (Fig. 1). As most Bcl-xL expressing cells were S100-positive, we investigated Bcl-xL upregulation in the SCs. As expected, we found a higher Bcl-xL expression in the nNF96.2 MPNST-derived SCs than in normal HSCs (Fig. 2). The anti-apoptotic Bcl-2 family member protein Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl1/A1 and Bcl-B, bind to and inactivate BH3-domain pro-apoptotic proteins (45). High expression levels of these proteins have been found in various types of cancer and have been related to the development of chemoresistance in malignant tumor cells (45-47). In our study, we found that increased Bcl-xL expression, but not that of Bcl-2 or Mcl-1, caused an increase in resistance to doxorubicin in MPNST-derived SCs (Figs. 2 and 3). Manipulating Bcl-xL expression levels demonstrated that the reduced apoptosis sensitivity of MPNST-derived SCs was caused by Bcl-xL overexpression (Fig. 3).

Although NF1 LOH has been identified in benign neurofibromas (21-24), a much higher frequency of NF1 LOH (3-4-fold) has been observed in MPNSTs compared to neurofibromas in patients with NF1 (25). The interaction between NF1+ SCs and other types of NF1− cells, including fibroblasts, mast cells and perineurial cells and the elevated expression of stem cell factors in NF1− SCs in the tumor microenvironment has been implicated in the tumor progression of PNs to MPNSTs (12,48). These results strongly indicate that the bi-allelic inactivation of the NF1 gene in SCs plays a crucial role in MPNST pathogenesis and NF1 tumorigenesis; however, little is known about the molecular mechanisms involved. Notably, NF1 deficiency promotes carcinogenesis by inducing heat shock factor 1 (HSF1), which is mediated by aberrant Ras/MAPK signaling (49). HSF1 overexpression and activation has been observed in NF1-deficient MPNST cells and tumor sections from patients with NF1 (49). Intriguingly, as shown in a previous study, NF1 deficiency contributes to the epithelial-mesenchymal transition (EMT) in NF1 (37). The expression levels of the EMT-related transcription factors, Snail, Twist and ZEB1, were significantly upregulated in the sNF96.2 MPNST-derived SCs compared with the normal HSCs. EMT is involved in cancer metastasis via the Ras/MAPK signaling pathway (50). Therefore, we investigated whether NF1 deficiency is directly involved in Bcl-xL upregulation in MPNST SCs. The results following manipulation of NF1 expression levels demonstrated a close correlation between neurofibromin and Bcl-xL levels and sensitivity to doxorubicin-induced apoptosis in sNF96.2 SCs and HSCs (Figs. 4 and 5). Taken together, these results indicate that the high Bcl-xL expression in MPNST-derived SCs is caused by NF1 deficiency.

Neurofibromin depletion by NF1 siRNAs in HSCs resulted in Erk1/2 activation (Fig. 4A) and an increase in BCL2L1 mRNA levels (Fig. 4B). Treatment with the Erk1/2 inhibitor, PD98059, resulted in a slight increase in Bcl-xL levels in the neurofibromin-depleted HSCs (Fig. 6). These results demonstrate that neurofibromin-mediated Bcl-xL expression is controlled at the transcriptional level via the Ras/MAPK signaling pathway. BCL2L1 gene transcription is regulated by a number of transcription factors, including Ets-1, Ets-2, Rel/nuclear factor-κB, signal transducers and activators of transcription, activator protein 1 and Spl (51-53). Of note, these proteins are all downstream of the Ras-signaling pathway (54). NF1 deficiency-mediated Ras activation has been identified in a subpopulation of SCs (NF1−) but not fibroblasts (NF1+) in mice with neurofibromas (55). Our results suggest that a neurofibromin deficiency in SCs caused by the bi-allelic inactivation at the NF1 locus enhanced Ras signaling, which consequently led to the expression of BCL2L1 transcription factors.

Bcl-xL overexpression contributes to the inhibition of the effects of many chemotherapeutic drugs (46,47). Reducing Bcl-xL expression restored apoptosis sensitivity to doxorubicin in sNF96.2 cells (Fig. 3), leading to a reasonable therapeutic strategy for patients with NF1 and MPNSTs through increased chemosensitization of malignant SCs by modulating the Bcl-xL expression level. ABT-737 selectively inhibits Bcl-2, Bcl-xL and Bcl-w (41). ABT-737 has been demonstrated to enhance synergistic chemosensitization when used in combination with doxorubicin in other MPNST cells (33), chordrosarcoma cells (56), and hepatoblastoma cells (57). We thus examined apoptosis sensitivity of sNF96.2 MPNST-derived SCs by the combined treatment of ABT-737 and doxorubicin. As a result, ABT-737 synergistically enhanced sensitivity to doxorubicin-induced apoptosis in sNF96.2 cells (Fig. 7). A very low dose of ABT-737 enhanced the cytotoxic effect of doxorubicin, and the concentrations required for approximately 50% cytotoxicity in sNF96.2 cells were 0.5 µg/ml doxorubicin and 0.1 µM ABT-737. Notably, the concentrations of ABT-737 and doxo-
rubricin required for effective apoptotic cell death were much lower in sNF96.2 cells than those in sNF02.2 cells, another MPNST SC line (33). sNF96.2 SCs are a NF1 LOH strain (NF1/−), whereas sNF02.2 SCs harbor one intact NF1 allele (NF1+/−). Considering that NF1+/− SCs play a major role in MPNST pathogenesis in NF1 (31,48) and that a high frequency of NF1− SCs is detected in NF1-associated MPNST tissues (25), ABT-737 and doxorubicin may increase the additive effects of the combined treatment in NF1− SCs. Considering that NF1+/− SCs play a major role in MPNST pathogenesis in NF1 (31,48) and that a high frequency of NF1− SCs is detected in NF1-associated MPNSTs (25), ABT-737 and doxorubicin seems to be a good combination to effectively treat NF1-associated MPNSTs with minimal side-effects. Although surgical resection is the primary treatment for MPNSTs, its limitation due to tumor location and tumor multiplicity has led to the development of a drug treatment approach. The proteins involved in the EGFR/Ras signaling and mTOR pathways have been the main chemotherapeutic targets for MPNSTs (6,58). In our study, increased cell survival caused by the prevention of apoptosis was closely related to the chemoresistance in NF1-associated MPNSTs, suggesting that Bcl-xL may be good candidate for MPNST-targeted drug treatment.

In conclusion, we found the overexpression of the anti-apoptotic protein, Bcl-xL, in MPNST tissues from patients with NF1 and in SCs derived from patients with NF1-associated MPNSTs. Our results demonstrate that the upregulation of Bcl-xL in MPNST-derived SCs was caused by NF1 deficiency-mediated elevation in Ras/MAPK signaling. Our findings may provide an opportunity for the development of novel chemotherapeutic strategies for patients with NF1 and MPNSTs.

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References


