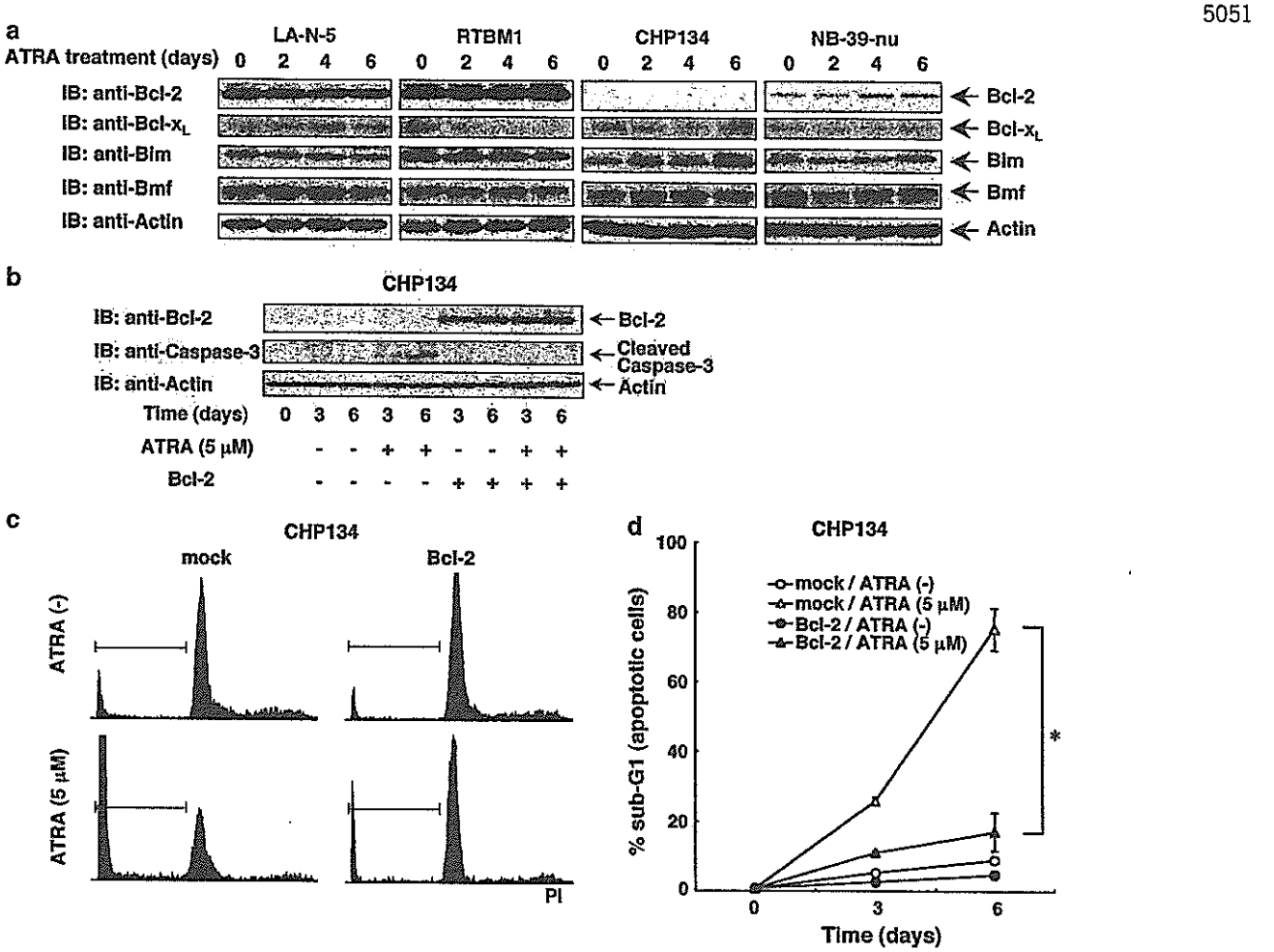

Figure 3 Differential expression of antiapoptotic Bcl-2 protein. (**a**) The indicated neuroblastoma cell lines were cultured in standard culture medium containing all-*trans* retinoic acid (ATRA) at a final concentration of 5 μM. At the indicated time points after the treatment with ATRA, whole-cell lysates were prepared, and analysed by immunoblotting with the antibodies against the indicated Bcl-2 family proteins. Actin expression was examined as a loading control (bottom). (**b**) Overexpression of Bcl-2. CHP134 cells were transiently transfected with the expression plasmid for Bcl-2 or with the empty plasmid. Twelve hours after the transfection, cells were treated with or without 5 μM ATRA, and incubated for additional 3 or 6 days. Whole-cell lysates were prepared, and the expression levels of Bcl-2 (top) and the amounts of the cleaved caspase-3 (middle) were examined by immunoblotting. Actin is shown as a control for protein loading (bottom). (**c** and **d**) Flow cytometry. CHP134 cells were transiently transfected as described in (**b**). At the indicated time periods after the treatment with ATRA, cells were collected, fixed and stained with PI. The DNA content of the cells was examined by flow cytometry. Representative results on day 6 are shown in (**c**). The number of cells with sub-G1 DNA content was counted in triplicate (**d**). *$P<0.01$.

1997; van Noesel and Versteeg, 2004). However, certain neuroblastomas display an RA-resistant phenotype (Reynolds and Lemons, 2001). To further improve the therapeutic effects of RA on neuroblastomas, it is necessary to clarify the detailed molecular mechanisms underlying the RA-mediated neuroblastoma differentiation and/or apoptosis. In the present study, we have found that ATRA causes growth suppression and subsequent neuronal differentiation in human neuroblastoma-derived LA-N-5, RTBM1, CHP134 and NB-39-nu cells to various degrees. Among them, CHP134 and NB-39-nu cells, which express antiapoptotic Bcl-2 at extremely low levels, underwent p53-independent apoptotic cell death in response to ATRA. In contrast, LA-N-5 and RTBM1 cells abundantly expressed Bcl-2, and we did not detect apoptotic cell death upon ATRA treatment. Enforced expression of Bcl-2 in CHP134 cells inhibited the ATRA-mediated apoptosis, and HA14-1-mediated inhibition of the endogenous Bcl-2 in RTBM1 cells enhanced the ATRA-dependent apoptotic cell death. Moreover, studies using primary neuroblastoma tissues showed that ATRA had toxic effect on two out of 10 primary cultures, and these ATRA-sensitive tumors did not express *bcl-2*. Thus, it is likely that antiapoptotic Bcl-2 plays a crucial role in the regulation of the ATRA-mediated apoptotic response in neuroblastomas.

Our present study revealed that neuroblastoma cells can be divided into two groups with respect to the ATRA-induced apoptotic response. CHP134 and NB-39-nu cells underwent apoptotic cell death in response to ATRA, whereas LA-N-5 and RTBM1 cells did not. Consistent with the mitochondria-dependent intrinsic apoptotic pathway of caspase activation (Degterev

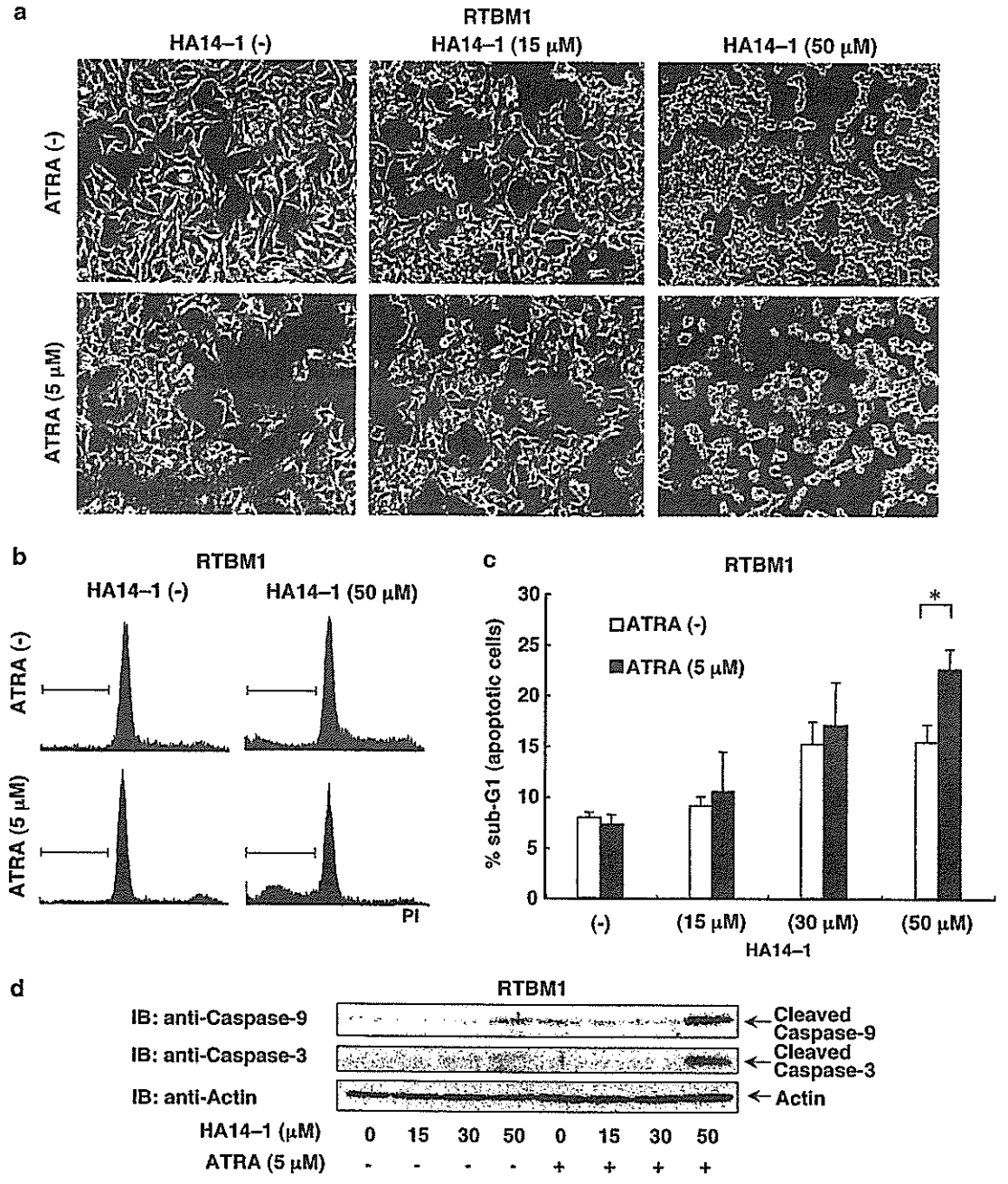

Figure 4 Bcl-2 inhibitor HA14-1 induces apoptosis of all-*trans* retinoic acid (ATRA)-treated RTBM1 cells. (a) Morphological changes after HA14-1 treatment of RTBM1 cells. RTBM1 cells were cultured with or without 5 μM ATRA for 6 days in advance, and then treated with HA14-1, a specific inhibitor of Bcl-2, at the indicated concentrations in standard medium for 3 h. Cells were examined by phase-contrast microscopy and photographed after the treatment. (b and c) FACS analysis. RTBM1 cells were treated with ATRA and HA14-1 as described in (a). Cells were collected, fixed and stained with PI. The DNA content of the cells was examined by flow cytometry and representative results are shown in (b). The number of cells with sub-G1 DNA content was counted in triplicate (c). $^*P<0.05$. (d) Immunoblotting. Whole-cell lysates of RTBM1 treated with ATRA and HA14-1 were prepared to examine the amounts of cleaved caspase-9 and caspase-3. Actin is shown as a loading control.

et al., 2003), ATRA treatment in CHP134 cells caused a cytoplasmic release of the mitochondrial inter-membrane protein cytochrome *c*, and a sequential proteolytic cleavage of caspase-9, caspase-3 and its physiological substrate PARP. Similar results were obtained in NB-39-nu cells. Our previous observation also demonstrated that activation and nuclear translocation of caspases were associated with prognosis of primary neuroblastomas (Nakagawara *et al.*, 1997). Therefore, the molecular mechanism(s) of RA-induced activation of caspases in neuroblastoma cells needs to be clarified.

In response to a variety of apoptotic stimuli, p53 is induced to be stabilized and subsequently transactivates a number of proapoptotic genes that encode Bcl-2

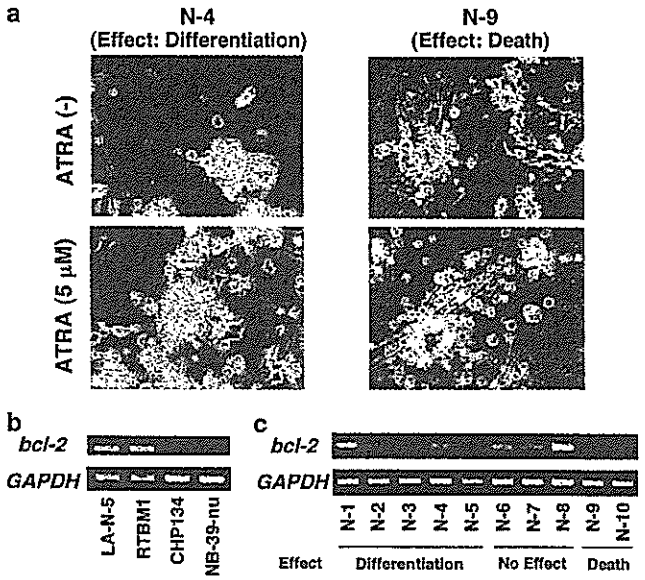

Figure 5 Primary culture and *bcl-2* expression of sporadic neuroblastomas. (a) Primary tumor cells prepared from 10 sporadically found neuroblastoma tissues were cultured with or without 5 μM of ATRA. After 4 days, cells were examined by phase-contrast microscope, and morphological changes of two representative cases are shown. (b and c) RT–PCR analysis. Total RNA was purified from the indicated neuroblastoma cell lines (b) and fresh-frozen tissues of primary neuroblastomas (c), and subjected to RT–PCR using specific primers for *bcl-2*. N-1 to N-10 indicates the case numbers, and the effects of ATRA on primary cultures are described at the bottom of this panel. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression is shown as an internal control.

family proteins including Bax (Culmsee and Mattson, 2005). It has been well documented that Bax acts on the mitochondria to induce mitochondrial permeability transition, and thereby regulating the cytoplasmic release of cytochrome *c* (Antonsson, 2001). Neuroblastoma cell lines that we examined in this study carry wild-type p53. Under our experimental conditions, however, we could not detect the ATRA-mediated upregulation of the endogenous p53 as well as Bax. Similarly, the p53-responsible $p21^{WAF1}$ and proapoptotic Puma were not accumulated in response to ATRA. As described (Nikolaev *et al*., 2003), p53 might not be functional in neuroblastoma cells due to its abnormal cytoplasmic retention. Consistent with this notion, p53 was predominantly expressed in the cytoplasm of neuroblastoma cells examined in this study (data not shown). We have previously shown that cytoplasmic p53 is translocated into the nucleus of CHP134 cells in response to ATRA (Takada *et al*., 2001); however, our present results suggest that translocated p53 was not functional. Indeed, it is reported that p53 in neuroblastoma cells is not functional even after its enforced translocation into the nucleus (Ostermeyer *et al*., 1996). Thus, it is likely that the ATRA-mediated apoptotic cell death in neuroblastoma cells is regulated in a p53-independent manner.

Among other regulators of mitochondrial pathway of apoptosis, Bcl-2 family proteins are critical determinants of mitochondrial membrane potential, which controls the cytoplasmic release of cytochrome *c* from mitochondria and thereby regulating apoptotic cell death (Cory *et al*., 2003). They are divided into two subfamilies based on their biological roles. Antiapoptotic subfamily includes Bcl-2 and Bcl-x_L and proapoptotic subfamily includes Bax, Bim and Bmf. The balance between these two groups determines the fate of cells. Antiapoptotic Bcl-2 is one of the most important members that inhibits the mitochondria-dependent apoptotic pathway triggered by diverse cytotoxic agents through blocking mitochondrial permeability transition. Indeed, the upregulation of Bcl-2 was associated with the drug-resistant phenotype of certain human tumors (Dole *et al*., 1994; Lombet *et al*., 2001). Most intriguingly, our expression studies revealed that antiapoptotic Bcl-2 was constitutively overexpressed in LA-N-5 and RTBM1 cells, whereas its expression levels were extremely low in CHP134 and NB-39-nu cells. In response to ATRA, Bcl-2 was slightly induced to be accumulated in NB-39-nu cells; however, it was maintained at extremely low levels in CHP134 cells. Furthermore, two primary neuroblastomas on which ATRA had toxic effect in primary culture did not express *bcl-2*, similar to CHP134 and NB-39-nu cells. Interestingly, ATRA induced differentiation in five cases and had undetectable effects on three cases, but cell death was induced in two cases. Considering that RA treatment contributed to survival of 17% of patients with aggressive neuroblastomas (Matthay *et al*., 1999), our present results using primary neuroblastomas seem to be reliable. Taken together, it is likely that ATRA potentially have toxic effect on certain neuroblastoma cells (both primary cells and cell lines) that express little Bcl-2. Our current results also revealed that enforced expression of Bcl-2 in CHP134 cells inhibited the ATRA-mediated apoptosis in association with the activation of caspase-3. Furthermore, ATRA treatment of RTBM1 cells followed by HA14-1 exposure underwent apoptotic cell death through mitochondrial pathway. These observations also support the importance of Bcl-2 in the regulation of apoptotic response of neuroblastoma cells to RA.

Although it is still unclear whether the expression levels of Bcl-2 could be correlated with the prognosis of neuroblastoma patients (Romani and Lu, 1994; Gallo *et al*., 2003; Abel *et al*., 2005), it is possible that Bcl-2 plays a key role at least in part in the regulation of ATRA-mediated apoptotic cell death in neuroblastoma cells. In this connection, the antisense RNA-mediated knockdown of the endogeneous Bcl-2 has been employed to treat certain tumors (Kim *et al*., 2004). Recently, a novel Bcl-2 inhibitor that has an antitumor effect on solid tumors has been developed (Oltersdorf *et al*., 2005). Based on our present findings, the combination of RA with Bcl-2-specific inhibitor might provide a novel therapeutic strategy for the treatment of neuroblastomas, instead of the classical chemotherapy that frequently has multi-organ side effects.

Materials and methods

Cell lines and transfection

Human neuroblastoma-derived cell lines, including LA-N-5, RTBM1, CHP134 and NB-39-nu, were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 in the air. For transfection, CHP134 cells were transfected with the expression plasmid encoding human Bcl-2 or with the empty plasmid by electroporation using a Nucleofector (Amaxa Biosystems, Koeln, Germany) according to the manufacturer's protocol.

Reagents

All-*trans* retinoic acid was purchased from Sigma (St Louis, MO, USA) dissolved in dimethylsulfoxisde (DMSO) at a final concentration of 5 mM, and kept at −80°C. Bcl-2 inhibitor HA14-1 was purchased from Sigma, dissolved in DMSO as a 10 mM stock solution and stored at −20°C. All reagents were of the highest quality available.

Proliferation assay

LA-N-5 and RTBM1 cells were plated in triplicate at a density of 1×10^5 per well in 12-well culture plates. CHP134 and NB-39-nu cells were seeded in triplicate at a density of 1×10^4 in 12-well plates. Twelve hours after seeding the cells, cells were treated with ATRA at a final concentration of 5 μM or left untreated, and medium was replaced every 2 days. At the indicated time points after the treatment with ATRA, cells were trypsinized and number of viable cells was directly scored by using the hemocytometer.

Flow cytometric analysis

Cells were exposed to the indicated concentration of ATRA. At the indicated time points after the treatment with ATRA, cells were collected by brief centrifugation, and fixed with 70% ethanol at −20°C. The cells were washed with phosphate-buffered saline (PBS), resuspended in phosphate-citrate buffer (4 mM citric acid, 200 mM Na_2HPO_4) and kept at room temperature for 15 min. The cells were then centrifuged and resuspended in a solution containing 40 μg/ml of propidium iodide and 0.05% RNase A, and incubated in the dark for 30 min. Before performing flow cytometric analysis, cells were filtered through a 40-μm nylon mesh. DNA content was analysed by FACScan flow cytometer (Becton Dickinson, Oxford, UK).

Immunoblot analysis

Cells were washed twice with ice-cold PBS, lysed in a sodium dodecyl sulfate (SDS)-sample buffer containing 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS and 62.5 mM Tris-HCl, pH 6.8, and then boiled for 3 min. The protein concentrations were determined using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin (BSA) was used as a standard. Aliquots (20 μg) of whole-cell lysates were separated by SDS–polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were blocked with 0.3% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 and incubated with appropriate primary antibodies at room temperature for 1 h followed by incubation with the horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology Inc., Beverly, MA, USA). Immunoreactive bands were visualized by using ECL system (Amersham Biosciences, Uppsala, Sweden). The primary antibodies used in this study were as follows: polyclonal anti-caspase-12 (Cell Signalling Technology Inc.), polyclonal anti-caspase-3 (Calbiochem, San Diego, CA, USA), polyclonal anti-PARP (Cell Signaling Technology Inc.), polyclonal anti-PUMA (ab9643; Abcam, Cambridge, UK), polyclonal anti-$p21^{WAF1}$ (H-164; Santa Cruz Biotechnology), polyclonal anti-Bim (Cell Signaling Technology Inc.), polyclonal anti-Bmf (Cell Signaling Technology Inc.), polyclonal anti-actin (20-33; Sigma), monoclonal anti-caspase-8 (5F7; Medical & Biological Laboratories, Nagoya, Japan), monoclonal anti-caspase-9 (5B4; Medical & Biological Laboratories), monoclonal anti-p53 (DO-1; Oncogene Research Products, Cambridge, MA, USA), monoclonal anti-Bax (6A7; eBioscience, San Diego, CA, USA), monoclonal anti-Bcl-2 (100; Santa Cruz Biotechnology); and monoclonal anti-Bcl-x_L (H-5; Santa Cruz Biotechnology) antibodies.

Immunofluorescent staining

CHP134 cells were grown on coverslips in standard culture medium in the presence or absence of 5 μM of ATRA for 4 days. Cells were washed with ice-cold PBS, fixed with 3.7% formaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min and then blocked with 3% BSA in PBS for 1 h at room temperature. After blocking, cells were incubated with a monoclonal antibody against cytochrome *c* (6H2.B4; BD PharMingen, San Jose, CA, USA) or with a normal mouse IgG for 1 h at room temperature, followed by the incubation with fluorescein isothiocyanate-conjugated secondary antibody against mouse IgG (Santa Cruz Biotechnology). The cell nuclei were stained with DAPI. The coverslips were mounted onto glass slides, and the stained cells were examined by using a confocal laser scanning microscope (Olympus).

Primary culture

RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and 100 μg/ml of OPI (Sigma) was used as a standard medium for primary culture. Primary tumor cells were prepared from fresh human neuroblastoma tissues by a standard method. A total of 5×10^5 cells of each sample were resuspended in 1 ml of the standard medium, and seeded on 24-well tissue culture plates precoated with collagen. The cells were treated with or without ATRA at a final concentration of 5 μM for at least 2 weeks. The effects of ATRA on the growth and neurite extension of primary neuroblastoma cells were examined by phase-contrast microscope.

RNA extraction and RT–PCR

Total RNA was prepared from fresh-frozen tissues of primary neuroblastomas or cultured cells by using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA (2 μg) was reverse transcribed by using random primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The resultant cDNA was subjected to PCR-based amplification. The oligonucleotide primers used in this study were as follows: *bcl-2*, 5′-GAGGATTGTGGCCTTCTTTG-3′ (forward) and 5′-ACAGTTCCACAAAGGCATCC-3′ (reverse), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5′-ACCTGACCTGCCGTCTAGAA-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). PCR products were electrophoretically separated on 1% neutral agarose gels and visualized by ethidium bromide staining.

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