Aloe vera Extract Suppresses Proliferation of Neuroblastoma Cells *In Vitro*

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Abstract. *Background/Aim: Neuroblastoma is a pediatric solid tumor refractory to eradication by chemotherapy. To determine whether Aloe vera (AV), a pontial anticancer reagent, could be useful in neuroblastoma therapy, we investigated the anti-proliferative effects of an AV protein extract. Materials and Methods: Human neuroblastoma cell lines (IMR-32, TGW, CHP-126 and NBL-S) were cultured with AV protein extract and proliferation status was assessed by cell counting, Ki-67 staining and gene expression. Results: Among tested lines, the number of viable, AV-treated IMR-32 cells significantly decreased 1.98-fold by day 2 and 1.33-fold by day 5 of culture relative to untreated controls ($p<0.05$). Treatment also decreased the number of Ki-67(+) IMR-32 cells by 13% by day 5 ($p<0.05$) and, unlike untreated controls, CCND2 mRNA expression levels became undetectable by day 1. Conclusion: AV-protein extract suppresses human IMR-32 neuroblastoma cell proliferation, possibly by suppressing CCND2 transcript levels in vitro.*

Neuroblastoma, a pediatric solid tumor derived from precursors of sympathetic nervous system, is the third most common cancer in children behind leukemia and brain tumors (1). Average annual incidence of neuroblastoma was 22.3 and 29.8 cases per 100,000 births in the US (1973-1992) and Japan (1980-1998) (2, 3), respectively. Presently, neuroblastoma is treated by a combination of surgery, radiation therapy and intensive chemotherapy. However, 60-70% of neuroblastoma patients who have poor prognosis, such as chromosome duplication and older age of onset, are resistant to chemotherapy (4) and more than 60% of chemotherapy-treated patients show disease recurrence (5). In addition, chemotherapy-related death accounts for 2-12% of patients (5). Therefore, development of more effective treatments for this condition are critical.

Plants are a natural source of bioactive molecules. *Aloe vera* (AV) is a succulent plant species used in traditional herbal medicine to treat burns. Recently, it was reported that AV also possesses anticancer activity (6-8). AV components, such as the emodin or aloe-emodin, reportedly suppress proliferation of human breast cancer cells (9), gastric cancer cells (10), human hepatoma cells (11) and glioma cells (12) *in vitro* and *in vivo* (13) (Table I). However, little is known about the anti-proliferative effects of AV in the case of neuroblastoma. To address this issue, we investigated the effect of an AV protein extract on human neuroblastoma cell proliferation. We report that this treatment suppressed proliferation of one human neuroblastoma cell line *in vitro*.

Materials and Methods

Preparation of an Aloe vera protein extract. The AV protein extract used in this study was provided by Natural Rendez-Vous Co., Ltd. (Ho Chi Minh City, Vietnam). The protein fraction of the extract was concentrated using an Amicon® Ultra Centrifugal Filter (molecular weight cut-off=3,000 Da, Millipore, Billerica, MA, USA). Protein quantity was measured using Quick Start™ Bradford Dye reagent (BIO-RAD, Hercules, CA, USA).

Cell culture and counting. Four human neuroblastoma lines were used in this study, including IMR-32 (14), TGW (15), CHP-126 (16) and NBL-S (17), provided from the Institute of Molecular

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Key Words: Neuroblastoma, *Aloe vera*, proliferation.

Table I. *Effect on human cancer cell lines of non-protein components of Aloe vera extracts.*

Cancer type	Cell type	Aloe	References
Leukemia	K562, HL-60, KG-1a, U937	Aloe-emodin	Mahbub AA *et al*., 2013 (33)
	K562, HL-60, U937	*Aloe vera* (DEHP)	Lee KH *et al*., 2000 (34)
	K562, HL-60, U937, THP-1	*Aloe vera* (DEHP)	Lee KH *et al*., 2000 (35)
Neuroblastoma	SJ-N-KP, SK-N-BE(2c)	Aloe-emodin	Pecere T *et al*., 2003 (8)
Breast	MCF-7	Aloe dawei	Abdissa N *et al*., 2014 (36)
	MCF-7	Emodin, Aloe-emodin	Huang PH *et al*., 2013 (9)
Prostate	PC-3	Aloe-emodin	Liu K *et al*., 2012 (9)
Lung	H460	Aloe-emodin	Lee HZ *et al*., 2010 (37)
	H460	Aloe-emodin	Chang WT *et al*., 2012 (38)
Tongue	SCC-4	Aloe-emodin	Chen YY *et al*., 2010 (39)
	SCC-4	Aloe-emodin	Chiu TH *et al*., 2009 (6)
Liver	Hep G2, Hep 3B	Aloe-emodin	Kuo PL *et al*., 2002 (11)
	Huh-7	Aloe-emodin	Jeon W *et al*., 2012 (40)

Embryology and Genetics, Kumamoto University (Kumamoto, Japan). IMR-32 cells were maintained in Eagle's minimum essential medium (EMEM) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) and 10 U/ml penicillin and 10 mg/ml streptomycin (Sigma-Aldrich, Saint Louis, MO, USA). TGW, CHP-126 and NBL-S cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries) supplemented with 10% FBS and 10 U/ml penicillin and 10 mg/ml streptomycin. All lines were cultured in a 37oC humidified atmosphere of 5% CO_2. Cells were passaged every 3-4 days. These cells were cultured with or without AV protein extract (0.5 μg/ml) and then seeded at 2.5×10^4 cells per well in 96-well plates. On days 1, 2 or 5, cells were harvested by treatment with 0.05% trypsin-EDTA (Wako Pure Chemical Industries) for 5 min at 37˚C and dissociated by pipetting. The number of viable cells was determined by trypan blue staining and counted under a microscope. Cells were cultured in triplicate wells for each cell line and means and standard deviations (SD) were calculated.

Immunocytochemistry. Cultured cells were attached onto glass slides (Matsunami glass, Osaka, Japan) by CytoSpin4 (Thermo Fisher scientific, Waltham, MA, USA) at 450 rpm for 7 min and air-dried thoroughly. Cells were fixed in PBS containing 1% paraformaldehyde at room temperature for 30 min, washed with PBS, blocked with PBS containing 1% BSA at room temperature for 30 min and incubated overnight at 4˚C with mouse anti-human Ki67 (1:500; eBioscience, San Diego, CA, USA) primary antibody. After 3 PBS washes, cells were incubated with goat anti-mouse IgG AlexaFluor488 (1:400; Life Technologies, Palo Alto, CA, USA) and TOTO-3 iodide (1:1500; Invitrogen, Carlsbad, CA, USA) at room temperature for 30 minutes. After 3 PBS washes, cells were mounted on coverslips with fluorescent mounting medium (Dako Corporation, Glostrup, Denmark) and assessed using a FluoView 1000 confocal microscope (Olympus, Tokyo, Japan). The percentage of Ki67-expressing cells was calculated from three different areas on the slide.

RNA extraction and real-time polymerase chain reaction (PCR). Total RNA was extracted from cultured cells using an RNAqueous®-4PCR Kit (Life Technologies) and mRNA was reverse transcribed into cDNA using the High-Capacity RNA-to-cDNAs™ Kit (Life Technologies). Expression of *CCND1, CCND2, BAX, BCL-2, MCL-1* and *ACTB* mRNA was assessed by StepOnePlus™ real-time PCR (Life Technologies) with Fast SYBR® Green Master Mix (Life Technologies). Transcript levels were normalized to *ACTB* mRNA and relative expression of each gene was calculated. Primer sequences were: *CCND1*, forward: 5'-CCACAGATGTGAAGTTCATTTCCA-3', reverse: 5'-AAGCGTGTGAGGCGGTAGTAG-3'; *CCND2*, forward: 5'-TTCTTCTTCCAAATGCAGTTCATT-3', reverse: 5'- TGCCTCCG TTTCATGTGAGTT-3'; *BAX*, forward: 5'-GGACGAA CTGGACA GTAACATGG-3', reverse: 5'-GCAAAGTAGAAAAGG GCGACAAC-3'; *BCL-2*, forward: 5'-ATCGCCCTGTGGATGAC TGAG-3', reverse: 5'-CAGCCAGGAGAAATCAAACAGAGG-3'; *MCL-1*, forward: 5'-CGGGCAAATCCTCCAAAAG-3', reverse: 5'- CCCTGAGAGAA GCGTAAGACAAA-3'; and *ACTB*, forward: 5'- ATTGCCGACA GGATGCAGA-3', reverse: 5'-GAGTACTTGCGC TCAGGAGGA-3'.

Statistical analysis. Results are presented as means±standard deviation (SD). To calculate statistical significance between non-treated control and AV protein extract-treated samples, we used Student's *t*-test. All *p*-values <0.05 were considered statistically significant.

Results

Effect of Aloe vera treatment on neuroblastoma cell proliferation. To investigate potential effects of an AV protein extract on proliferation, we cultured four human neuroblastoma lines IMR-32, TGW, CHP-126 and NBL-S with extract for a maximum of 5 days and assessed their proliferative status at various time points. In all four lines, the total number of viable cells in non-treated and extract-treated samples gradually increased by day 5. However, in IMR-32 cells, the total number of viable cells was significantly lower in the AV protein extract-treated sample relative to non-treated controls at day 2 (0.51-fold, $p<0.05$) and day 5 (0.75-fold, $p<0.01$) (Figure 1A). By contrast, we observed comparable numbers of viable cells when we cultured TGW (Figure 1B), CHP-126 (Figure 1C) or NBL-S (Figure 1D) cells with or without extract. To further examine the effect of AV protein extract on IMR-32 cells, we assessed the morphology of treated and untreated cells microscopically.

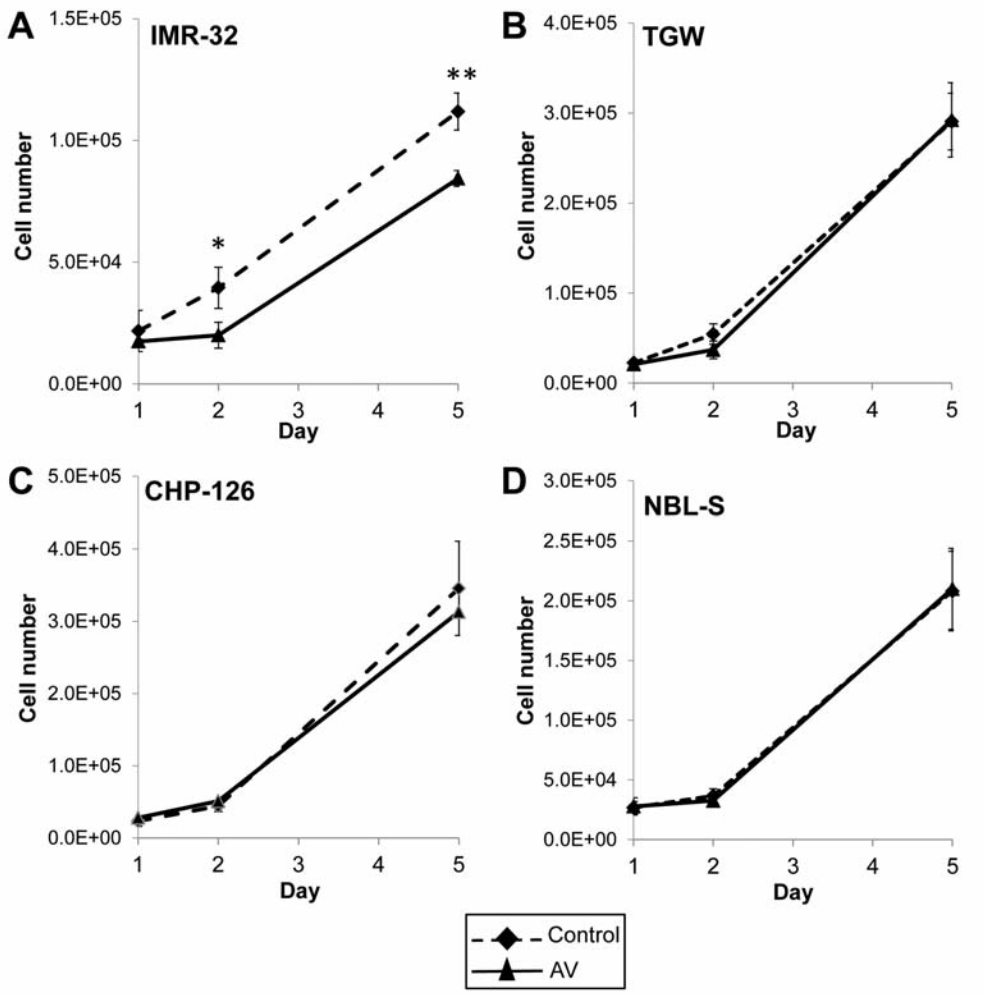

Figure 1. *Proliferation of human neuroblastoma cell lines in the presence of Aloe vera (AV) protein extract. Four human neuroblastoma lines (IMR-32, TGW, CHP-126 and NBL-S) were cultured with the extract and viable cells were counted using trypan blue staining on days 1, 2 and 5. The total number of viable IMR-32 (A), TGW (B), CHP-126 (C) and NBL-S (D) cells is shown (n=3). *p<0.05, **p<0.01.*

As shown in Figure 2, low and high magnifications of the microscopic images were obtained to examine cell density and morphology, respectively. IMR-32 cells became confluent by day 5 in the presence or absence of AV protein extract. Examination of both cultures revealed the presence of neuroblast-like cells, which lack neurite extension. Morphological differences were not evident between conditions.

To confirm the decreased proliferation of IMR-32 cells in the presence of extract, we performed immunocytochemical staining for Ki-67, a nuclear marker of proliferating cells (Figure 3A) (18, 19). To calculate the percentage of Ki-67(+) cells, we counted total number of TOTO3(+) cells and compared them with Ki-67+ cells. At days 1 and 2, the percentage of Ki-67(+) cells did not differ significantly between AV protein extract-treated samples (93.6±3.0% and 97.9±1.9%, respectively) and non-treated controls (84.9±6.5% and 92.0±3.1%, respectively) (Figure 3A and 3B). However, in agreement with decreases observed in the total number of viable cells, by day 5 the percentage of Ki-67(+) cells had decreased 12.3±3.12% in extract-treated cells (74.7±4.6%) compared to that seen in non-treated controls (87.0 ±2.9%) (Figure 3B, $p<0.05$).

Aloe vera protein treatment suppresses expression of cell proliferation-related genes. To investigate the mechanisms underlying the effects of AV protein extract on cell proliferation, we examined the transcript levels of cell proliferation-related genes, such as *CCND1* and *CCND2*, using real-time PCR (20). As shown in Figure 4A, we observed no changes in *CCND1* transcript levels at days 1 or 2 in extract-treated or untreated controls, whereas, by day 1 of culture, *CCND2* expression decreased to undetectable levels in AV protein extract-treated cultures (Figure 4A, right). Relative

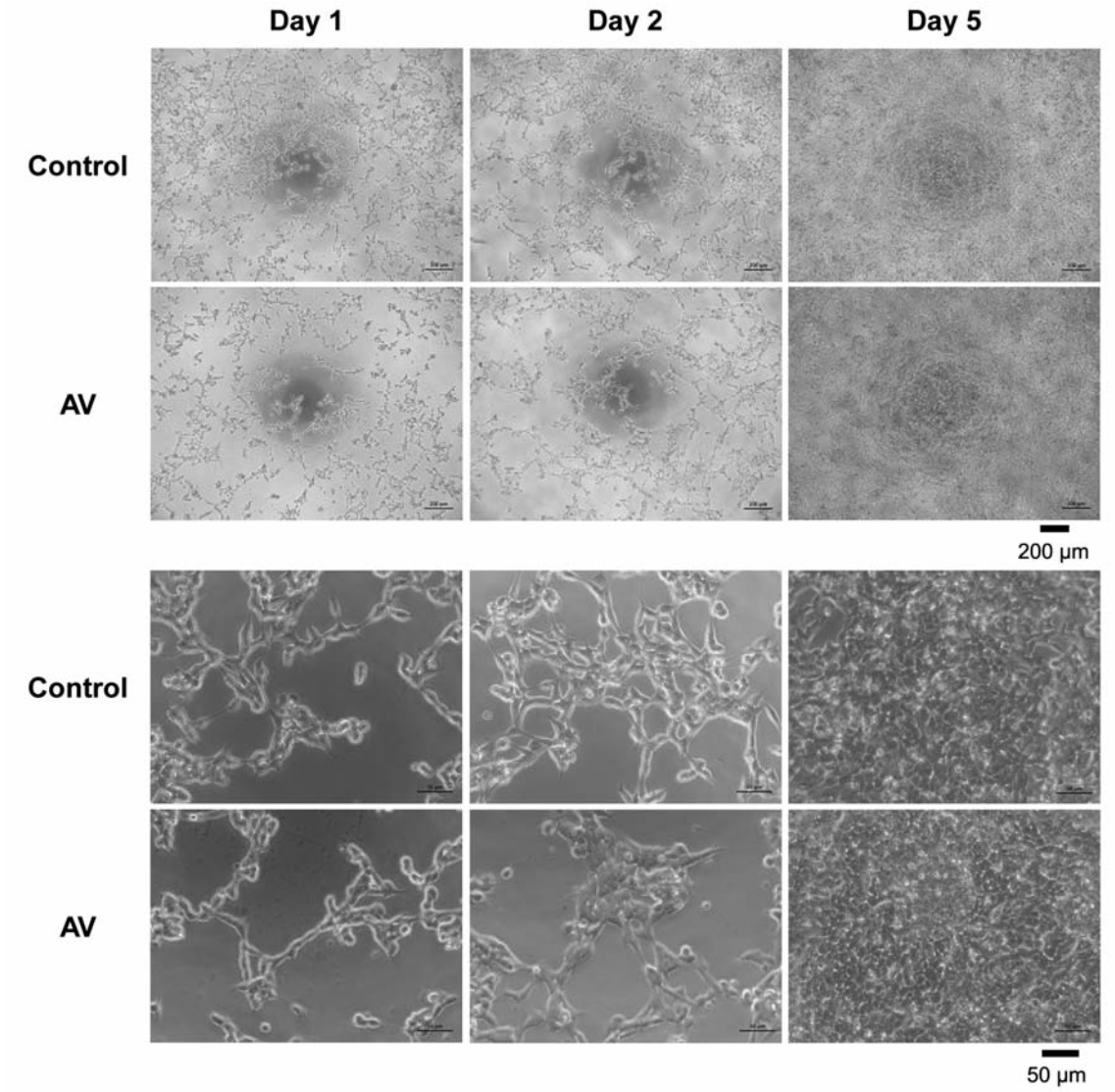

Figure 2. *Morphology of IMR-32 cells cultured with Aloe vera (AV) protein extract. Microscopic images of IMR-32 cells cultured with or without extracts are shown. Low and high magnifications of the microscopic images show cell density and morphology, respectively. Scale bars: 200 μm in upper panels and 50 μm in lower.*

down-regulation of *CCND2* mRNA was also observed at day 2 in extract-treated samples (1.8-fold decrease).

To determine whether the decrease in number of viable IMR-32 cells seen in the presence of AV protein extract was due to apoptosis, we assessed expression of the apoptosis-related genes *BAX* (21) and *BCL-2* (22) and the cell survival-related gene *MCL-1* (23) using samples taken at day 1 of culture. As shown in Figure 4B, we observed no significant differences in the relative expression of any of these transcripts between non-treated controls and AV extract-treated samples, suggesting that decreases in cell number seen in extract-treated samples are primarily due to altered proliferation.

Discussion

Herein, we report the effect of AV protein extract on proliferation of IMR-32 human neuroblastoma cells *in vitro*. In patients, neuroblastoma originates in sympathetic nervous system precursors in regions from neck to pelvis, including thorax, retroperitoneum, abdomen, adrenal gland, kidney and pelvis. Among affected tissues, neuroblastoma most frequently occurs in the adrenal gland, followed by the retroperitoneum and abdomen (24). We assessed proliferation of IMR-32, CHP-126 and NBL-S cells, that are derived from the abdomen, retroperitoneum and adrenal

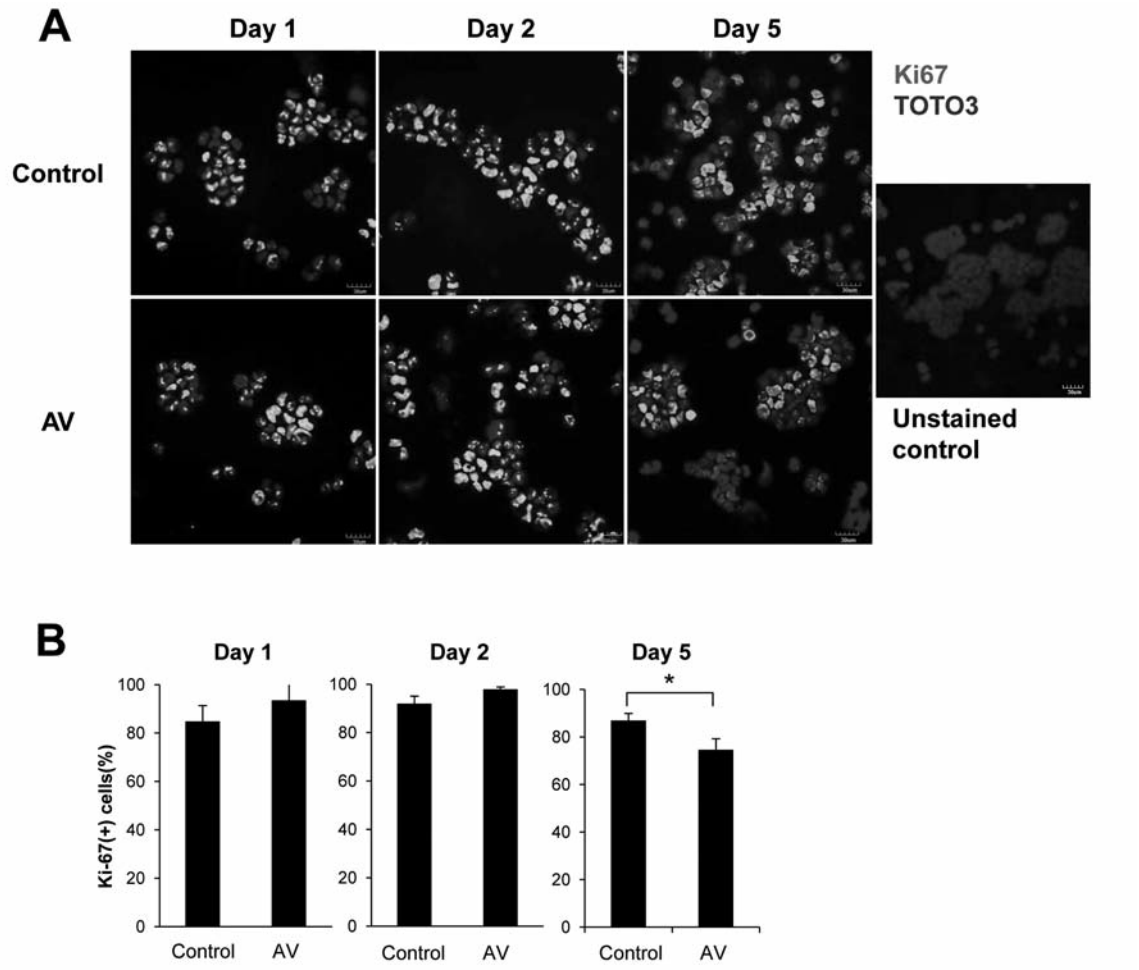

Figure 3. *IMR-32 cell proliferation in the presence of Aloe vera (AV) protein extract. (A) Representative confocal images showing nuclear Ki-67 staining of IMR-32 cells cultured with AV protein extract for 1, 2 or 5 days. Ki-67 (green) staining is located in the nucleus (blue), which is stained with TOTO3. Scale bars: 30 μm for all panels. (B) Percentage of Ki-67(+) cells among total TOTO3(+) cells. Ki-67(+) cells were counted in 2-3 different fields and captured by confocal microscopy.*

gland, respectively, as well as of TGW cells, that are derived from nude mice xenografted with human neuroblastoma cells of adrenal gland. We show that treatment with AV protein extract suppressed human IMR-32 cell proliferation *in vitro*, possibly through *CCND2* down-regulation. Cytogenetically, neuroblastoma cells often show deletion or translocation of the distal portion of the short arm of chromosome 1 (chromosome 1p), which harbors tumor suppressor genes. The status of chromosome 1p differs among neuroblastoma lines: IMR-32 cells show 1p deletion, whereas CHP-126 cells exhibit a translocation in that region.

Ki-67 is expressed in the nucleus of proliferating cells in G_1, S, G_2 and M phases but is absent in the non-proliferative G_0 phase of the cell cycle (18, 19, 27, 28). A decrease in the number of Ki-67(+) cells following treatment with AV protein extract indicates suppressed cell proliferation. Lack of change in genes related to apoptosis supports the idea that components of the extract suppress proliferation rather than increase apoptosis.

c-MYC, CCND1 and CCND2 are cell-cycle regulators whose expression is positively correlated with cell proliferation (29). We assessed *c-MYC* mRNA expression in all neuroblastoma lines tested but observed no or barely detectable levels (data not shown). Thus, we further evaluated only *CCND1* and *CCND2* mRNA expression. In adult mice, loss-of-function *CCND2* mutations abolish proliferation of neuronal precursors (30). In addition, among D-type cyclins (*CCND1, CCND2* and *CCND3*), only *CCND2* mRNA is reportedly expressed in dividing neuronal precursors. This finding suggests that *CCND2* plays a major role in neuronal cell proliferation.

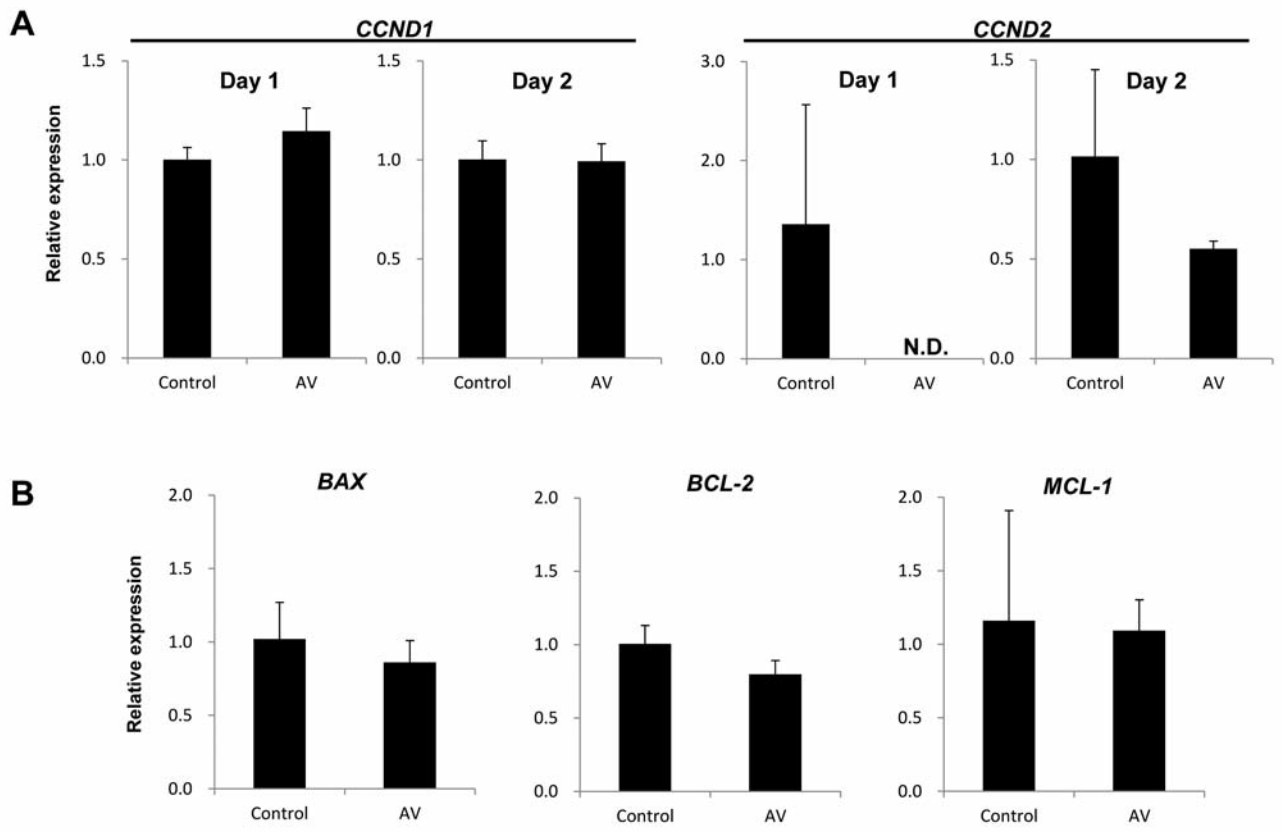

Figure 4. *Gene expression analysis of IMR-32 cells cultured with Aloe vera (AV) protein extract. (A) IMR-32 cells were cultured in the presence of extract for 1 and 2 days and assessed for relative expression of CCND1 (left) and CCND2 (right) transcripts by real-time polymerase chain reaction (PCR). CCND2 expression was undetectable (N.D.) in extract-treated cells at day 1. (B) Expression of apoptosis-related genes (BAX and BCL-2) and the cell survival-related gene MCL-1 was assessed by real-time PCR. mRNA levels were normalized to ACTB mRNA and shown as relative expression.*

Proteins extracted from AV reportedly have immuno-stimulatory activity (31, 32). For example, in mice, ATF1011, a non-mitogenic lectin purified from the leaves of *Aloe arborescens* Miller, stimulates anti-tumor immunity by activating T lymphocytes (32). The glycoprotein aloctin A, derived from the leaves of *Aloe arborescens*, has a prophylactic effect on Ehrlich ascites tumor in mice (31). However, the effect of AV protein extracts on cancer cell proliferation has been unclear. Herein, we demonstrated an anti-proliferative activity of AV protein extract on a human neuroblastoma cell line. Further proteomic-based identification of protein components in crude protein extracts will enable us to define mechanisms underlying AV anti-proliferative activity, efforts that could lead to development of novel therapies for neuroblastoma.

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