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N-Myc knockdown and apigenin treatment controlled growth of malignant neuroblastoma cells having N-Myc amplification

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Abstract

Malignant neuroblastomas mostly occur in children and are frequently associated with N-Myc amplification. Oncogene amplification, which is selective increase in copy number of the oncogene, provides survival advantages in solid tumors including malignant neuroblastoma. We have decreased expression of N-Myc oncogene using short hairpin RNA (shRNA) plasmid to increase anti-tumor efficacy of the isoflavonoid apigenin (APG) in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cell lines that harbor N-Myc amplification. N-Myc knockdown induced morphological and biochemical features of neuronal differentiation. Combination of N-Myc knockdown and APG most effectively induced morphological and biochemical features of apoptotic death. This combination therapy also prevented cell migration and decreased N-Myc driven survival, angiogenic, and invasive factors. Collectively, N-Myc knockdown and APG treatment is a promising strategy for controlling the growth of human malignant neuroblastoma cell lines that harbor N-Myc amplification.

Keywords

apigenin; apoptosis; differentiation; neuroblastoma; N-Myc shRNA

1. Introduction

Malignant neuroblastoma, which is an embryonic tumor derived from primitive cells of the sympathetic nervous system, is one of the most common and deadly solid tumor in infants and children (Maris et al., 2007). Despite multimodal therapeutic approaches including chemotherapy, radionuclide therapy, and immunotherapy, the survival rate for patients with malignant neuroblastoma remains poor. Newer therapeutic strategies are urgently needed for successful treatment of this devastating childhood malignancy.

N-Myc is a member of the Myc oncogene family that also includes cellular Myc. This oncogene as amplified DNA with homology to viral Myc was first discovered in human

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neuroblastoma cell lines and a neuroblastoma tumor (Schwab et al., 1983). Myc family genes are frequently deregulated in numerous types of human cancer. Whereas cellular Myc is expressed in a wide variety of human tumors, N-Myc expression is mostly restricted to the tumors of the nervous system, such as neuroblastoma (Adhikary and Eliers, 2005). N-Myc amplification has also been reported in retinoblastoma (Lee et al., 1985) and small-cell-lung carcinoma (Nau et al., 1986). High N-Myc expression is known to occur in 20 – 22% neuroblastomas at diagnosis and 40% progressive neuroblastomas (Cohn and Tweddle, 2004). The increased N-Myc expression has been correlated with activation of the oncogenes that promote tumor aggression (Norris et al., 1996). High N-Myc activity and low level of neuronal differentiation have been associated with poor outcome in malignant neuroblastoma (Fredlund et al., 2008). Low level of neuronal differentiation in neuroblastoma might also be due to the presence of the surrounding hypoxic microenvironment (Jögi et al., 2002). Hypoxic microenvironment is known to foster the increases in Notch ligands and inhibitor of differentiation 2 (ID2) in the neural crest region resulting in low level of neuronal differentiation in neuroblastoma cells (Löfstedt et al., 2004). Amplified expression of N-Myc has been extensively associated with high malignant behavior and poor prognosis of neuroblastoma (Schwab, 2004). After all, N-Myc amplification remains to be the most important and reliable oncogenic marker that strongly correlates with advanced stages of this malignancy and poor survival of the patients. Presumably, N-Myc promotes progression of neuroblastoma through regulation and/or cooperation with other oncogenic pathways. However, the exact nature of these pathways remains largely unclear.

Failure of the current therapeutic regimens to inhibit the N-Myc related angiogenic factors continues to contribute to high proliferation and invasion of neuroblastoma cells, resulting in poor prognosis of the neuroblastoma patients. These facts call for the need to develop new therapeutic strategies using gene silencing and/or combination of different drugs. In our present study in human malignant neuroblastoma cells having N-Myc amplification, we inhibited the expression of N-Myc gene by using the N-Myc short hairpin RNA (shRNA) plasmid encoding N-Myc cDNA and also treatment with apigenin (APG), a well-known dietary isoflavonoid present in fruits and vegetables. APG induces apoptotic death in many cancers, including human malignant neuroblastoma (Mohan et al., 2013). Phytochemicals such as flavonoids and isoflavonoids have been highly regarded for their anti-tumor effects on multiple human malignancies (Chen et al., 2007). Dietary isoflavonoids including APG play major roles in modulating a number of key elements in signal transduction pathways in cancers (Ramos, 2008).

In this study, we showed efficacy of combination of the N-Myc knockdown and APG treatment in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cell lines that harbor N-Myc amplification. Combination therapy was more efficacious than monotherapy to induce apoptosis through activation of both extrinsic and intrinsic pathways in SK-N-DZ and SK-NBE2 cells. Our study also demonstrated that combination of N-Myc knockdown and APG treatment controlled the growth of these malignant neuroblastoma cells by modulating the expression of the molecules involved cell differentiation, migration, angiogenesis, and invasion.

2. Materials and methods

2.1. Cell culture conditions

The human malignant neuroblastoma SK-N-DZ (amplified N-Myc and wild-type p53), SKN-BE2 (amplified N-Myc and mutant p53), SH-SY5Y (single copy N-Myc and wild-type p53), and IMR32 (amplified N-Myc and wild-type p53) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). SK-N-DZ and IMR32

cell lines were maintained in DMEM medium while SK-N-BE2 and SH-SY5Y cell lines were maintained in RPMI 1640 medium, both supplemented with 10% fetal bovine serum (FBS) (Atlanta Biological, Atlanta, GA, USA) and 1% antibiotics, and allowed to in a humidified incubator containing 5% CO₂ at 37°C. N-Myc shRNA plasmid and control shRNA plasmid (encoding scrambled shRNA sequence) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). APG was obtained from Sigma Chemical (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide to make stock solution and aliquots of stock solution were stored at –20 °C until ready to use.

2.2. Immunofluorescence microscopy to examine expression of N-Myc in malignant neuroblastoma cell lines

Laser scanning confocal immunofluorescence microscopy was performed according to our previously described method (Hossain et al., 2012) with slight modification. Briefly, cells were grown on the poly-D-lysine chamber slides (BD Biosciences, Bedford, MA, USA) at 37°C in presence of 5% CO₂ for 24 h. Cells in the slide were washed twice in phosphate-buffered saline (PBS, pH 7.4, without Ca²⁺ and Mg²⁺) followed by fixation in 4% formaldehyde in PBS for 30 min at room temperature. After fixation cells were washed in PBS and made permeable with 0.25% Triton X-100 in PBS for 10 min. Cells were washed and non-specific binding were blocked by 3% bovine serum albumin (BSA) in PBS for 10 min. Primary IgG antibody against N-Myc was diluted (1:200) in 1% BSA in PBS. After the incubation with primary IgG antibody for 1 h at room temperature, cells were washed twice with PBS and stained with fluorescein isothiocyanate (FITC) conjugated anti-rabbit secondary IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) in 1% BSA in PBS for 1 h at room temperature and washed again with PBS. To counter stain the nucleus, cells were then incubated with 300 nM 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Eugene, OR, USA) for 5 min and washed four times before mounting with Dabco 33-LV (Sigma Chemical, St. Louis, MO, USA). The nuclear stain DAPI could strongly bind to A-T rich regions in DNA to show blue fluorescence. Fluorescence images were captured using Zeiss LSM 510 META confocal microscope (Zeiss, Germany) and analyzed by Zeiss LSM image browser software.

2.3. Flow cytometry to monitor the expression of N-Myc in malignant neuroblastoma cell lines

Adherent cells were detached from culture flasks by incubating with 0.02% EDTA (Sigma Chemical, St. Louis, MO, USA) in PBS and then dissociated into monodispersed cells. Cells (1×10^5) were collected and suspended in 100 µl of 1× flow cytometry buffer (1% BSA and 0.1% NaN₃ in PBS, filtered through the 0.22 µm filter unit) containing an FcBlocker (eBioscience, San Diego, CA, USA) and incubated at 4°C for 15 min. Then, primary IgG antibody against N-Myc (1:200) was added to the cell suspension. Parallel staining was also performed with rabbit IgG (1:200) (Jackson ImmunoResearch, West Grove, PA, USA) as an isotype matched control for N-Myc. After incubation at 4°C for 30 min, cells were washed twice with 1× flow cytometry buffer. Cells were then incubated in 100 µl of 1× flow cytometry buffer containing FITC conjugated goat anti-rabbit secondary IgG antibody (1:200) at 4°C for 30 min. Then, cells were washed as above, fixed in 2% paraformaldehyde in PBS, and transferred to a 5-ml polystyrene round-bottom tube capped with a cell-strainer 183 cap (BD Biosciences, Franklin Lakes, NJ, USA). Antibody-bound cells were then analyzed in a cytometer using CellQuest software (BD Biosciences, Bedford, MA, USA) and FlowJo software (Tree Star, Ashland, OR, USA).

2.4. Transfection with N-Myc shRNA plasmid and treatment with APG

Both SK-N-DZ and SK-N-BE2 cell lines were grown at 37°C in 5% CO₂ and full-humidity. Both cell lines were transfected with the mammalian expression vector carrying N-Myc

shRNA cDNA or scrambled shRNA cDNA, treated with 50 μ M APG, or combination of N-Myc shRNA plasmid and 50 μ M APG in medium containing 2% FBS. The concentration of APG was chosen on the basis of our previous study (Mohan et al., 2011a). Cells were transfected with 1 μ g plasmid in 6-well dishes using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions and incubated at 37°C in 5% CO₂. After 6 h, transfection reagent was replaced by fresh medium containing 2% FBS. After 18 h, the medium was again replaced with fresh medium only or fresh medium containing 50 μ M APG and incubated for another 24 h. The transfection efficiency was measured by counting the percentage of green-fluorescent cells.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) to examine N-Myc mRNA

The RT-PCR experiments were conducted to monitor the knockdown of expression of NMyc mRNA in cells after transfection with the N-Myc shRNA plasmid. Both SK-N-DZ and SK N-BE2 cell lines were grown at 37°C in 5% CO₂. Both cell lines were transfected with N-Myc shRNA plasmid or scrambled shRNA plasmid, treated with 50 μ M APG, or combination of NMyc shRNA plasmid and 50 μ M APG in the growth medium containing 2% FBS. After the treatments, cells were used to isolate total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Following primer sequences were used for PCR amplification of N-Myc gene (forward: 5'-ACC ACA AGG CCC TCA GTA CC-3' and reverse: 5'-GTG CAT CCT CAC TCT CCA CG-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (forward: 5'-ATG GGG AAG GTG AAG GTC GG-3' and reverse: 5'-AGA CGC CAG TGG ACT CCA CGA CG-3'). The cDNA was synthesized using SuperScript one-step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) in a thermal cycler (Eppendorf, Westbury, NY, USA) at 50°C for 30 min followed by 30 cycles of PCR amplification (denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min) and final extension 72°C for 10 min. The RT-PCR products were resolved by electrophoresis on 1% agarose gels, stained with ethidium bromide (1 μ g/ml), and visualized using a UV chamber (Alpha Innotech, San Leandro, CA, USA). Expression of GAPDH mRNA was used as an internal standard.

2.6. In situ methylene blue staining to examine morphology of neuronal differentiation

After transfection with N-Myc shRNA plasmid or scrambled shRNA plasmid, cells were cultured in 6-well dishes for 3 days. The transfected cells were washed twice with ice-cold PBS in the culture plate followed by fixation with 2 ml of ice-cold 95% (v/v) ethanol for 5 min. After fixation, we aspirated ethanol, washed the cells twice with PBS, and then stained with 2 ml of ice-cold 0.2% (v/v) methylene blue solution (prepared in 50% ethanol) for 20 sec. After staining, the cells were washed with distilled water and air dried before taking photograph under the light microscope. The dimensions of the cells (length and width) and neurite length were measured (n = 20) using ImagePro Plus software version 4.5.1.29 (Media Cybernetics, Silver Spring, MD, USA).

2.7. In situ Wright staining for detection of morphological features of apoptotic death

After the treatments, both adherent and non-adherent cells were spun down at 3,500 rpm for 10 min. Cells were washed with PBS, fixed, and stained with HEMA3 stain according to the manufacturer's instruction (Fisher Scientific, Kalamazoo, MI, USA). Cells were allowed to dry after the staining and the morphological features of the cells were observed under the light microscope and digital pictures were captured. All the experiments were conducted in triplicates and representative pictures were presented.

2.8. Flow cytometry for detection of a biochemical feature of apoptosis

After the treatments, both adherent and non-adherent cells were collected in 15-ml tubes and washed twice with 10 ml of PBS. Cells were stained with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI), processed as per the manufacturer's instructions (BD Biosciences, SD, CA, USA), and then analyzed on Epics XL-MCL Flow Cytometer (Beckman Coulter, Fullerton, CA, USA), as we reported recently (Chakrabarti et al., 2013). PI positive and Annexin V-FITC negative cells were considered as mechanically injured (quadrant A1), both PI and Annexin V-FITC positive cells were considered as late necrotic (quadrant A2), both PI and Annexin V-FITC negative cells were considered as normal (quadrant A3), and PI negative and Annexin V-FITC positive cells were considered as early apoptotic (quadrant A4). Flow cytometry detected the Annexin V-FITC positive cells with externalization of membrane phospholipids, an early biochemical feature of apoptosis. All the experiments were conducted in triplicates and representative pictures were shown. The Annexin V-FITC stained apoptotic cells were analyzed for statistical significance.

2.9. Protein extraction

Cells were grown in 150-mm diameter dishes and subjected to transfection, treatment, or both as above before protein extraction. Cells were scraped into growth medium, collected into 15-ml tubes, and centrifuged to harvest the pellets. The cell pellets were washed twice with 20 ml of ice-cold PBS, suspended in 400 μ l of ice-cold homogenization solution (50 mM Tris-HCl, pH 7.4, 320 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA), transferred to eppendorf tubes, and subjected to sonication gently in micro-ultrasonic cell disruptor (Kontes, Vineland, NJ, USA). The cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C and the supernatants were collected. The protein concentrations in the supernatants were measured using Coomassie Plus protein assay reagent (Pierce Biotechnology, Rockford, IL, USA). All the samples were divided into small aliquots and stored at -20°C until they were used.

2.10. Western blotting using specific antibodies

Each protein sample (10 μ g) were mixed with Laemmli buffer and denatured in boiling water for 5 min. Then, all protein samples were loaded onto the precast 4–20% polyacrylamide gradient gels (Bio-Rad Laboratories, Hercules, CA, USA) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the SDS-PAGE, the resolved protein samples from the gels were electroblotted to the polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The primary IgG antibody against α -actin (clone AC-15) was purchased from Sigma Chemical (St. Louis, MO, USA) and primary IgG antibodies against NMyc, neurofilament protein (NFP), neuron specific enolase (NSE), e-cadherin, Notch-1, ID2, proliferating cell nuclear antigen (PCNA), human telomerase reverse transcriptase (hTERT), caspase-8, Bid, Bcl-2, Bax, calpain, caspase-3, spectrin breakdown product (SBDP), inhibitor of caspase-activated DNase (ICAD), p-Akt (Thr 308), p65 nuclear factor-kappa B (NF- κ B), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), matrix metalloproteinase-2 (MMP-2), and MMP-9 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The PVDF membranes or blots were incubated overnight at 4°C with an appropriate dilution of a primary IgG antibody followed by washing with a washing buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20). Then, blots were incubated with an appropriate alkaline horseradish peroxidase (HRP) conjugated secondary IgG antibody for 1 h followed by washing with washing buffer. The alkaline HRP conjugated anti-rabbit and antimouse secondary IgG antibodies were purchased from Biomed (Foster City, CA, USA) and anti-goat secondary IgG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Specific protein bands were detected by incubation with Immuno-Star™ HRP Lumino/Enhancer (Bio-

Rad Laboratories, Hercules, CA, USA) followed by autoradiography using BIOMAX XAR films (Kodak, Rochester, NY, USA). All experiments were performed in triplicates.

2.11. In vitro cell migration assay

In vitro cell migration assay, as we reported recently (Mohan et al., 2013), was performed to determine the effects of N-Myc shRNA plasmid or APG alone and combination of both on the migratory property of human malignant neuroblastoma cells. The migration assay was carried out in 6-well transwell inserts of polycarbonate membrane with 8.0 μm pore size (Corning, Lowell, MA, USA). The transwell inserts were coated with Matrigel (BD Biosciences, San Jose, CA, USA) of final concentration of 1.0 mg/ml in an ice-cold serum-free medium and allowed to dry at 37°C for 4 h. After the treatments, cells were trypsinized, washed twice with serum-free medium. Then, cell suspension (1×10^5 cells in 500 μl) from each sample was added to each transwell insert in triplicate. Cells were incubated at 37°C in presence of 5% CO_2 for 48 h and the membranes were collected and stained with DIFF quick stain kit (IMEB, San Marcos, CA, USA). The cells that migrated to undersurface of the membrane were observed using light microscope, photographed, and counted in 10 randomly selected microscopic fields.

2.12. Statistical analysis

Results from some of the experiments were analyzed for statistical significance using Minitab 16 statistical software (Minitab, State College, PA, USA). Data were expressed as mean $\pm$ standard deviation (SD) of separate experiments ($n = 3$) and compared by one-way analysis of variance (ANOVA) followed by the Fisher's post-hoc test. Difference between a control (CTL) group and a treatment group was considered significant at $p < 0.05$.

3. Results

3.1. In situ immunofluorescence microscopy and flow cytometry to examine expression of N-Myc in human malignant neuroblastoma cell lines

We used two different methods to examine the expression of N-Myc protein in four human malignant neuroblastoma SK-N-DZ, SK-N-BE2, IMR32, and SH-SY5Y cell lines (Fig. 1). For in situ detection of expression of N-Myc, we used laser scanning confocal immunofluorescence microscopy that showed expression of N-Myc protein at high level in SK-N-DZ and SK-N-BE2 cells, moderate level in IMR32 cells, and low level in SH-SY5Y cells (Fig. 1A). Further, we used flow cytometry for quantifying the levels of expression of this protein in these cell lines (Fig. 1B). The results from flow cytometric analysis revealed that levels of expression of N-Myc protein were 95%, 92%, 70%, and 37% in SK-N-DZ, SK-N-BE2, IMR32, and SH-SY5Y cells, respectively (Fig. 1B). Amplification of N-Myc gene is responsible of overexpression of N-Myc protein in SK-N-DZ and SK-N-BE2 cell lines. Our results (Fig. 1) confirmed that relatively overexpression of N-Myc protein occurred in SK-N-DZ and SK-N-BE2 cell lines, which were used in all subsequent experiments.

3.2. Knockdown of expression of N-Myc in SK-N-DZ and SK-N-BE2 cell lines

We examined the knockdown of expression of N-Myc at mRNA and protein levels in malignant neuroblastoma SK-N-DZ and SK-N-BE2 cell lines following scrambled shRNA plasmid transfection, N-Myc shRNA plasmid transfection, APG treatment, and combination of N-Myc shRNA plasmid transfection and APG treatment (Fig. 2). Our RT-PCR and Western blot analyses showed the amounts of N-Myc knockdown at mRNA (Fig. 2A) and protein (Fig. 2B) levels, respectively. When compared with untreated cells, transfection with the scrambled shRNA plasmid did not cause changes in mRNA and protein levels.

Transfection with N-Myc shRNA plasmid was more effective than treatment with APG for N-Myc knockdown at mRNA and protein levels. The amounts of N-Myc at mRNA and protein were most effectively down regulated due to combination of N-Myc shRNA plasmid transfection and APG treatment. RTPCR and Western blotting were carried out using, respectively, GAPDH mRNA expression and α -actin protein expression as the internal controls. Over 76% knockdown of expression of N-Myc at mRNA and protein levels occurred in both SK-N-DZ and SK-N-BE2 cell lines after combination of N-Myc shRNA plasmid transfection and APG treatment (Fig. 2C).

3.3. Induction of morphological and biochemical features of neuronal differentiation in SK-N-DZ and SK-N-BE2 cell lines following N-Myc knockdown

We monitored induction of neuronal differentiation in both malignant neuroblastoma SK-N-DZ and SK-N-BE2 cell lines following N-Myc shRNA plasmid transfection (Fig. 3). First, we monitored the morphological features of neuronal differentiation using the in situ methylene blue staining (Fig. 3A). N-Myc shRNA plasmid transfection was highly effective in inducing morphological features of neuronal differentiation in both cell lines (Fig. 3A). Neuronal differentiation was associated with small and retracted cell bodies having thin elongated and branched neurite extensions. Measurements of the cell width, cell length, and neurite length showed that N-Myc knockdown significantly shrunk the cell width but increased the cell length and the neurite length, when compared with cells transfected with the scrambled shRNA plasmid transfection (Fig. 3B). Subsequently, Western blotting showed the changes in biochemical markers of neuronal differentiation in SK-N-DZ and SK-N-BE2 cells (Fig. 3C). N-Myc knockdown and APG treatment increased expression of NFP, NSE, and e-cadherin (a tight junction protein), all of which served as the prominent biochemical markers of neuronal differentiation in the cells. Neuronal differentiation was also confirmed from inhibition of expression of Notch-1, ID2 (a member of the inhibitor of differentiation family), and hTERT (catalytic subunit of human telomerase) following N-Myc knockdown and APG treatment. Cells need to stop proliferation before induction of differentiation. Almost complete inhibition of expression of PCNA (a cell proliferation marker) promoted neuronal differentiation in the cells after N-Myc knockdown and APG treatment.

3.4. Induction of morphological and biochemical features of apoptosis in SK-N-DZ and SK-N-BE2 cell lines following N-Myc knockdown and APG treatment

An important goal of anti-cancer therapy is the induction of apoptotic death. So, we examined induction of morphological and biochemical features of apoptosis in both SK-N-DZ and SK-N-BE2 cells after N-Myc knockdown and APG treatment (Fig. 4). The in situ Wright staining showed the characteristic morphological features of apoptosis including shrinkage of cell volume, nuclear fragmentation, chromatin condensation and fragmentation, and membrane bound apoptotic bodies (Fig 4A). The arrows were used to show the apoptotic cells with at least one of these characteristic morphological changes, which occurred massively following N-Myc knockdown and APG treatment (Fig. 4A). N-Myc knockdown induced significant apoptosis in both neuroblastoma cell lines and the amounts of apoptosis were further increased with APG treatment (Fig. 4B). An early biochemical feature of apoptosis is externalization of membrane phospholipids that can be detected by Annexin V-FITC staining of the cells. Annexin V-FITC/PI staining and subsequent flow cytometry showed that Annexin V-FITC positive apoptotic population (A4 quadrant) were increased moderately in both SK-N-DZ and SK-N-BE2 cell lines after N-Myc knockdown or APG treatment alone (Fig. 4C). Combination of N-Myc knockdown and APG treatment dramatically increased Annexin V-FITC positive apoptotic population (A4 quadrant) in both cell lines (Fig. 4C). N-Myc knockdown and APG treatment caused the highest increase in induction of apoptotic death (Fig. 4D).

3.5. Combination of N-Myc knockdown and APG treatment activated both extrinsic and intrinsic caspase cascades for apoptosis in SK-N-DZ and SK-N-BE2 cell lines

Western blotting showed the changes in expression of proteins or activation of proteases for induction of both extrinsic and intrinsic pathways of apoptosis in both SK-N-DZ and SK-N-BE2 cell lines following N-Myc shRNA plasmid transfection or/and APG treatment (Fig. 5). Only N-Myc knockdown or APG treatment moderately induced extrinsic pathway of apoptosis as evidenced from activation of caspase-8. Combination of N-Myc knockdown and APG treatment further increased activation of caspase-8. Increase in proteolytic activity of caspase-8 was confirmed in the cleavage of Bid to tBid (Fig. 5), which could be translocated to mitochondria for augmentation of apoptosis via intrinsic pathway. Proteins of the Bcl-2 family play key roles in regulation of mitochondrial permeability for induction or inhibition of apoptosis via intrinsic pathway. N-Myc knockdown caused an increase in expression of pro-apoptotic Bax protein and decrease in expression of anti-apoptotic Bcl-2 protein in both cell lines (Fig. 5). Combination of N-Myc knockdown and APG treatment most effectively increased expression of Bax and decreased expression of Bcl-2, which could result in increase in the Bax:Bcl-2 ratio in both cell lines. We also examined the activation and activity of calpain and caspase-3 in both cell lines (Fig. 5). Calpain and caspase-3 were activated most prominently after combination of N-Myc knockdown and APG treatment. Proteolytic activities of calpain and caspase-3 greatly generated calpain-specific 145 kDa SBDP and caspase-3-specific 120 kDa SBDP, respectively, in both cell lines following N-Myc knockdown and APG treatment (Fig. 5). Caspase-3 activity also caused ICAD fragmentation, moderately after monotherapy and mostly after combination therapy, in both cell lines. The ICAD fragmentation could release of CAD from the CAD/ICAD complex and thereby translocation of CAD to the nucleus for DNA fragmentation.

3.6. Combination of N-Myc knockdown and APG treatment most effectively inhibited cell migration and down regulated molecules involved in cell survival, angiogenesis, and invasion pathways in SK-N-DZ and SK-N-BE2 cell lines

Efficacy of N-Myc knockdown and APG treatment was evaluated in inhibition of migration of malignant neuroblastoma SK-N-DZ and SK-N-BE2 cells (Fig. 6). We also examined the expression of molecules involved in survival, angiogenesis, and invasion pathways (Fig. 7). Cells that migrated through Matrigel were stained and examined using light microscope (Fig. 6A). The scrambled shRNA plasmid transfection did not inhibit cell migration but N-Myc knockdown or APG treatment moderately inhibited cell migration. Combination of N-Myc knockdown and APG treatment dramatically inhibited cell migration (Fig. 6A). We determined the amounts of cell migration found that combination of N-Myc knockdown and APG treatment most significantly inhibited migration of both SK-N-DZ and SK-N-BE2 cell lines (Fig. 6B). Subsequently, we performed Western blotting and demonstrated that N-Myc knockdown and APG treatment most effectively down regulated the molecules involved in cell survival (p-Akt and NF- κ B), angiogenesis (VEGF and b-FGF), and invasion (MMP-2 and MMP-9) pathways in both SK-N-DZ and SK-N-BE2 cell lines (Fig. 7).

4. Discussion

Our current study showed that combination of N-Myc knockdown and APG treatment very effectively induced neuronal differentiation and also extrinsic and intrinsic pathways of apoptosis in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cells harboring N-Myc amplification. Transition to neuronal phenotype was accompanied by increased expression of NFP, NSE, and the tight junction protein e-cadherin along with decreased levels of Notch-1, ID2, hTERT and PCNA. We also demonstrated for the first time that combination of N-Myc shRNA plasmid transfection and APG treatment led to the

suppression of survival factors (p-Akt and NF- κ B), angiogenic factors (VEGF and b-FGF), and invasive factors (MMP-2 and MMP-9) in these malignant neuroblastoma cell lines.

We determined relative levels of expression of N-Myc protein in four malignant neuroblastoma cell lines (SK-N-DZ, SK-N-BE2, IMR32, and SH-SY-5Y). SK-N-DZ and SK-N-BE2 cell lines, which harbored N-Myc gene amplification, highly expressed N-Myc protein. Selective inhibition of N-Myc induces differentiation and apoptosis in neuroblastoma cells (Pession et al., 2004). N-Myc prevents differentiation in neuroblastoma cells and its down regulation promotes differentiation in neuroblastoma cells (Rudie Hovland et al., 2001). We demonstrated dramatic down regulation of N-Myc in SK-N-DZ and SK-N-BE2 cells following N-Myc shRNA plasmid transfection and APG treatment.

A previous study used retrovirally delivered N-Myc shRNA to knockdown of N-Myc and examined only neuronal differentiation in human malignant neuroblastoma Kelly and SK-N-BE2 cell lines (Henriksen et al., 2011). On the other hand, our current study used plasmid mediated knockdown of N-Myc for induction of morphological features of neuronal differentiation, such as decrease in cell width and increases in cell length and neurite length, in human malignant neuroblastoma SK-N-DZ and SK-N-BE2 cell lines. We also examined the efficacy of combination of N-Myc knockdown and APG treatment in modulating the biochemical markers for induction of neuronal differentiation in these cell lines. The biochemical markers such as NFP and NSE, known to be upregulated during neuronal differentiation (Mohan et al., 2011b), were highly increased in SK-N-DZ and SK-N-BE2 cell lines following this combination therapy. Transition to neuronal phenotype was accompanied by substantial decreases in N-Myc and its downstream effectors such as Notch-1 and ID2, which could promote dedifferentiation in neuroblastoma cells (Fredlund et al., 2008). Our results also showed that neuroblastoma cells dramatically decreased expression of these dedifferentiation molecules after N-Myc knockdown and APG treatment. On the contrary e-cadherin, which could promote cell differentiation (Kim et al., 2005), was highly upregulated after N-Myc knockdown and APG treatment. Induction of neuronal differentiation was correlated with repression of cell proliferation markers such as hTERT and PCNA, as we reported previously (Janardhanan et al., 2009).

Our in situ Wright staining and also Annexin V-FITC staining demonstrated that N-Myc knockdown and APG treatment significantly increased amounts of apoptosis. Our Western blotting revealed the key molecules involved in extrinsic and intrinsic pathways of apoptosis. We found activation of caspase-8, which suggested induction of extrinsic pathway of apoptosis. Caspase-8 activity could cleave Bid to tBid for translocation of tBid to the mitochondrial membrane so as to aid release of several pro-apoptotic factors from mitochondrial inter membrane space (Luo et al., 1998; Kim, 2005). We found that N-Myc knockdown and APG treatment highly increased active caspase-8 for cleaving of Bid to tBid, confirming the induction of extrinsic pathway of apoptosis in SK-N-DZ and SK-N-BE2 cells.

Caspase-8 mediated cleavage of Bid to tBid and translocation of tBid to mitochondria provides a link between receptor and mitochondrial pathways of apoptosis (Fulda and Debatin, 2006). Cleavage of Bid to tBid could be associated with alterations in expression of proapoptotic Bax and anti-apoptotic Bcl-2 (Hossain et al., 2012). Increase in Bax:Bcl-2 ratio is an important indicator for triggering the mitochondrial release of various pro-apoptotic molecules into the cytosol to induce intrinsic pathway of apoptosis (Karmakar et al., 2006). Upregulation of various anti-apoptotic molecules including Bcl-2 protects the cancer cells from apoptosis (George et al., 2009). In this study, we showed that N-Myc knockdown and APG treatment caused upregulation of Bax with a concomitant down regulation of Bcl-2 so

as to cause an increase in the Bax:Bcl-2 ratio for promotion of intrinsic pathway of apoptosis in SK-N-DZ and SK-N-BE2 cells.

Mitochondria mediated activation of the effector caspases, including caspase-3, can cleave a number of cytoplasmic and nuclear substrates leading to completion of apoptotic process (Karmakar et al., 2006). Simultaneous increases in calpain and caspase-3 activities in human cancer cells in culture (Hossain et al., 2012) and animal (Karmakar et al., 2010) models promote apoptosis. Cytochrome c is released from mitochondria into cytosol and it binds to apoptosis-activating factor-1 (Apaf-1) to produce apoptosome leading to activation caspase-9 and then caspase-3 for the caspase-dependent apoptosis in cancer cells (Kim, 2005). We demonstrated that N-Myc knockdown and APG treatment dramatically increased activation of both calpain and caspase-3. Increased proteolytic activities of calpain and caspase-3 generated 145 kDa SBDP and 120 kDa SBDP, respectively. Also, caspase-3 activity caused cleavage of ICAD in both SKN-BE2 and SH-SY-5Y cell lines.

Inhibition of cell migration is an important goal of a successful anti-cancer therapeutic strategy in malignant neuroblastoma (Mohan et al., 2013). We found that combination therapy dramatically reduced cell migration in both neuroblastoma SK-N-DZ and SK-N-BE2 cell lines. N-Myc driven mitogenic and survival pathways significantly contribute to growth and invasiveness of malignant neuroblastoma (Misawa et al., 2000). Mitogenic and Akt survival pathways have the ability to intensify cellular proliferation, inhibit apoptosis, and potentiate the downstream NF- κ B survival pathway (Karin et al., 2002). Activation or phosphorylation of Akt (p-Akt) promotes cell survival and proliferation via activation of NF- κ B. N-Myc drives angiogenesis in tumor microenvironment due to production of the pro-angiogenic factors such as VEGF and b-FGF and also the invasive factors such as MMP-2 and MMP-9 (Chesler et al., 2007). We showed that combination of N-Myc knockdown and APG treatment very efficiently decreased survival factors (p-Akt and NF- κ B), angiogenic factors (VEGF and b-FGF), and invasive factors (MMP-2 and MMP-9) in both SK-N-DZ and SK-N-BE2 cell lines.

In conclusion, our current investigation demonstrated that combination of N-Myc knockdown and APG treatment resulted in significant increases in morphological and biochemical features of differentiation and apoptosis in human malignant neuroblastoma SK-NDZ and SK-N-BE2 cell lines harboring N-Myc amplification. We confirmed the modulation of expression of the molecular components for induction of differentiation and apoptosis in the cells. Combination of N-Myc knockdown and APG treatment also inhibited cell migration and survival, angiogenic, and invasive factors in these malignant neuroblastoma cells. Therefore, combination of N-Myc knockdown and APG treatment can be highly useful for controlling the growth of human malignant neuroblastoma cells that harbor N-Myc amplification.

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Abbreviations

APG	apigenin
b-FGF	basic fibroblast growth factor
BSA	bovine serum albumin
CTL	control
DAPI	4',6-diamidino-2-phenylindole
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HRP	horseradish peroxidase
hTERT	human telomerase reverse transcriptase
ICAD	inhibitor of caspase-activated DNase
ID2	inhibitor of differentiation 2
MMP-2	matrix metalloproteinase-2
NFP	neurofilament protein
NF- B	nuclear factor-kappa B

NSE	neuron specific enolase
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PI	propidium iodide
PVDF	polyvinylidene fluoride
RT-PCR	reverse transcription-polymerase chain reaction
SBDP	spectrin breakdown product
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
VEGF	vascular endothelial growth factor.

Research Highlights

- Human malignant neuroblastoma cells frequently harbor N-Myc amplification.
- N-Myc knockdown promotes differentiation in malignant neuroblastoma cells.
- The isoflavonoid apigenin (APG) induces apoptosis in malignant neuroblastoma cells.
- Combination of N-Myc knockdown and APG increases apoptosis in neuroblastoma cells.
- Combination of N-Myc knockdown and APG most effectively inhibited cell migration.

Fig. 1.

Detection and determination of relative expression of N-Myc protein in human malignant neuroblastoma cell lines. (A) Representative immunofluorescence staining to show expression of N-Myc (FITC, green) in human malignant neuroblastoma (SK-N-DZ, SK-N-BE2, IMR32, and SH-SY5Y) cell lines. DAPI (blue) was used staining the nucleus. (B) Flow cytometry for relative expression of N-Myc (FITC, green) in comparison with control (no stain, black).

Fig. 2.

Detection and determination of changes in expression of N-Myc at mRNA and protein levels in SK-N-DZ and SK-N-BE2 cell lines. Treatments: control (CTL), scrambled shRNA plasmid (0.5 µg/ml) for 48 h, N-Myc shRNA plasmid (0.5 µg/ml) for 48 h, 50 µM APG for 24 h, and N-Myc shRNA plasmid (0.5 µg/ml) for 48 h + 50 µM APG for last 24 h. (A) RT-PCR experiments for detection of changes in expression of N-Myc mRNA. Expression of GAPDH mRNA was used as an internal control. (B) Western blotting for detection of changes in expression of N-Myc protein. Expression of β-actin was used as a loading control. (C) Determination of changes in expression of N-Myc at mRNA and protein levels. Mean values of 3 independent experiments are shown with a significance such as * $p < 0.05$.

0.05, ** $p < 0.01$, or # $p < 0.05$ (* or ** when compared with CTL, # when compared with N-Myc shRNA or APG alone).

Fig. 3. N-Myc knockdown induced morphological and biochemical features of neuronal differentiation and inhibited cell proliferation in SK-N-DZ and SK-N-BE2 cell lines. (A) In situ methylene blue staining. Representative photographs were taken after transfection (72 h) with scrambled shRNA plasmid (0.5 μ g/ml) or N-Myc shRNA plasmid (0.5 μ g/ml). (B) Measurement of morphological features of neuronal differentiation. Mean values of 3 experiments are shown with a significance such as * $p < 0.05$ or ** $p < 0.01$ when compared with scrambled shRNA. (C) Western blotting for biochemical markers of neuronal differentiation and cell proliferation. Treatments: scrambled shRNA plasmid (0.5 μ g/ml) for 48 h, N-Myc shRNA plasmid (0.5 μ g/ml) for 48 h, 50 μ M APG for 24 h, and N-Myc shRNA

plasmid (0.5 µg/ml) for 48 h + 50 µM APG for last 24 h. Expression of β -actin was used as a loading control.

Fig. 4.

Determination of apoptosis in SK-N-DZ and SK-N-BE2 cell lines. Treatments: scrambled shRNA plasmid (0.5 $\mu\text{g/ml}$) for 48 h N-Myc shRNA plasmid (0.5 $\mu\text{g/ml}$) for 48 h, 50 μM APG for 24 h, and N-Myc shRNA plasmid (0.5 $\mu\text{g/ml}$) for 48 h + 50 μM APG for last 24 h. (A) In situ Wright staining for morphological features of apoptosis. (B) Bar diagrams to show apoptosis based on Wright staining. Mean values of 3 experiments were shown with a significance such as ** $p < 0.01$ (when compared with scrambled shRNA) or # $p < 0.05$ (when combination therapy was compared with N-Myc shRNA or APG alone). (C) Flow cytometry after Annexin V-FITC/PI staining. Apoptotic cells are shown in A4 quadrant. (D) Bar diagrams to show apoptosis based on accumulation of in A4 quadrant. Mean values of 3

experiments were shown with a significance such as $^{**}p < 0.01$ (when compared with scrambled shRNA) or $^{\#}p < 0.05$ (when combination therapy was compared with N-Myc shRNA or APG alone).

Fig. 5. Western blotting to examine molecules involved in induction of extrinsic and intrinsic pathways of apoptosis in SK-N-DZ and SK-N-BE2 cell lines. Treatments: scrambled shRNA plasmid (0.5 μ g/ml) for 48 h, N-Myc shRNA plasmid (0.5 μ g/ml) for 48 h, 50 μ M APG for 24 h, and N-Myc shRNA plasmid (0.5 μ g/ml) for 48 h + 50 μ M APG for last 24 h. Expression of β -actin was used as a loading control. All experiments were performed in triplicates.

Fig. 6. Cell migration assay in SK-N-DZ and SK-N-BE2 cell lines. Treatments: scrambled shRNA plasmid (0.5 µg/ml) for 48 h, N-Myc shRNA plasmid (0.5 µg/ml) for 48 h, 50 µM APG for 24 h, and N-Myc shRNA plasmid (0.5 µg/ml) for 48 h + 50 µM APG for last 24 h. (A) Inhibition of cell migration. After the treatments, cells were seeded for migration through the Matrigel coated membrane. (B) Quantitation of cell migration. Mean values of 3 experiments were shown with a significance such as * $p < 0.05$ or ** $p < 0.01$ (when with scrambled shRNA) or # $p < 0.05$ (when combination therapy was compared with N-Myc shRNA or APG alone).

Fig. 7.

Western blotting to examine the molecules involved in survival, angiogenesis, and migration of SK-N-DZ and SK-N-BE2 cell lines. Treatments: scrambled shRNA plasmid (0.5 μ g/ml) for 48 h, N-Myc shRNA plasmid (0.5 μ g/ml) for 48 h, 50 μ M APG for 24 h, and N-Myc shRNA plasmid (0.5 μ g/ml) for 48 h + 50 μ M APG for last 24 h. Expression of β -actin was used as a loading control. All experiments were performed in triplicates.