Expression of *URG4/URGCP*, *Cyclin D1*, *Bcl-2*, and *Bax* genes in retinoic acid treated SH-SY5Y human neuroblastoma cells

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Retinoic acid (RA) plays important roles in development, growth, and differentiation by regulating the expression of its target genes. The pro-apoptotic *Bax* gene may form channels through oligomerization in the mitochondrial membrane and facilitate the cytosolic release of cytochrome c. The anti-apoptotic *Bcl-2* gene can inhibit this process. Up-regulated gene 4/Upregulator of cell proliferation (*URG4/URGCP*) is a novel gene located on 7p13. *URG4/URGCP* also stimulates cyclin D1 (*CCND1*) mRNA expression, and RNAi-mediated *URG4/URGCP* silencing diminishes *CCND1* mRNA expression in HepG2 cells. In this study, the effects of RA treatment on *URG4/URGCP*, *CCND1*, *Bcl-2* and *Bax* gene expression changes in undifferentiated and differentiated SHSY5Y neuroblastoma cells was analyzed. SHSY5Y cells were cultured in the appropriate conditions. To induce differentiation, the cells were treated with 10 micromolar RA in the dark for 3-10 days. SHSY5Y cells possess small processes in an undifferentiated state, and after treatment with RA, the cells developed long neurites, resembling a neuronal phenotype. Total RNA was isolated with Tri-Reagent. Expression profiles of the target genes were determined by semi-quantitative RT-PCR. According to the results, *Bcl-2* and *CCND1* gene expression levels were increased, while *URG4/URGCP* and *Bax* gene expression was decreased in RA treated cells compared to the control cells. Our preliminary results suggest that RA may induce cell proliferation and escape apoptosis using a novel pathway by the *URG4/URGCP* gene. Further investigations are needed to clarify more direct transcriptional targets of RA signaling and the interaction of RA pathways with other pro-regenerative signals.

Key words: retinoic acid, *URG4/URGCP*, human neuroblastoma cells, SHSY5Y.

Contemp Oncol (Pozn) 2013; 17 (4): 346–349
DOI: 10.5114/wo.2013.34634

Introduction

Neuroblastoma (NB) is the most frequent extra-cranial solid malignant tumor found in childhood cancers [1]. Approximately half of all children with NB are classified as high-risk patients, despite intensive therapeutic cures. In view of these cases, any effort to improve diagnosis and therapy is of great clinical interest.

Retinoids are involved in the proliferation, differentiation, and apoptosis of various cell types. Active retinoids occur in three forms: alcohol (retinol), aldehyde (retinal or retinaldehyde), and acid (retinoic acid – RA). Retinoic acid plays important roles in development, growth, and differentiation by regulating the expression of its target genes. Retinoic acid appears to directly regulate more than 500 proteins [2–4]. The role of retinoids as agents inducing differentiation has been under investigation, and their use in the diet remains a promising therapy for the prevention of several types of cancer [5–7]. Neuronal differentiation can be induced *in vitro* in NB cells by exposure to RA, which is the most commonly used compound for differentiation. Sidell determined that RA can induce growth inhibition and morphologic differentiation of human NB cells *in vitro* in 1982 [8]. The differentiating inducer RA can drive the cells in different directions during their maturation. Additionally, it is discussed that the dramatically different response to RA is dependent on the tumor stage of the patient. Thus, aggressive and spreading tumor cells could be transformed *in vitro* to mature and non-proliferating cells [9, 10].

Apoptosis is a complex biological mechanism that organisms use to eliminate unwanted cells. Specialized cellular receptors and signals initiate the apoptotic machinery and a complex set of reactions leads to characteristic changes. Inhibition of apoptosis in the tumor cells would lead to cellular immortality. The Bcl-2 family of proteins has expanded significantly and includes both pro- as well as anti-apoptotic molecules. Bcl-2 family proteins are important regulators of apoptosis. They consist of a wide variety of anti-apoptotic proteins such as Bcl-2, but also include pro-apoptotic proteins such as Bax. Bcl-2 has been shown to promote cell survival. Bax, a pro-apoptotic protein member of the Bcl-2 family, participates in the induction of apoptosis in response to many apoptotic signals. Over-expression of Bax has been shown to induce apoptosis in a variety of different cellular contents [11, 12]. Bcl-2 is a pro-survival protein that resides on the mitochondrial membrane and prevents apop-

tosis through its effects upon the activation of the proteases necessary for the process. The presence of an anti-apoptotic molecule such as Bcl-2 or Bcl-xl can inhibit the activation of Bax following a death signal [13]. In contrast to inactive Bax, which is monomeric and in the cytosol or loosely associated with membranes, Bcl-2 is an integral membrane protein heavily localized to mitochondria.

In the past decade, a very large number of proto-oncogenes and tumor-suppressor genes have been found. In spite of the sizable number of genes already described, new genes with oncogenic potential or tumor suppressing activity are still being identified. Recently, *URG4/URGCP*, a novel gene upregulated by HBxAg in human hepatocellular carcinoma (HCC), has been identified (GenBank accession no. NM_017920) [14]. *URG4/URGCP* is located on chromosome 7 (7p13). Previous data suggested that overexpression of *URG4/URGCP* in HepG2 cells promoted hepatocellular growth and survival in tissue culture and nude mice. Hence, *URG4/URGCP* may be an oncogene that contributes to multistep hepatocellular carcinogenesis. Other studies have also suggested that *URG4/URGCP* might be an oncogene operating in gastric cancer and osteosarcoma. *URG4/URGCP* was found to be associated with PCNA labeled index in gastric cancer tissues, and overexpression of *URG4/URGCP* protein in GES-1 and HepG2 stimulates cell growth and promotes the entry of the cells into S-phase through cell cycle related protein *CCND1*. *CCND1* is a key factor in the cell cycle that functions between G0/G1- and S-phase check points. It is frequently over-expressed in HCC patients with enhanced malignant phenotypes and high mortality. Previous publications revealed that HBx up-regulates *CCND1* promoter in Chang liver and HepG2 cells, which may have an important role in the HBx-mediated HCC development and progression [15]. Over-expression of *URG4/URGCP* stimulated *CCND1* mRNA expression, and RNAi-mediated *URG4/URGCP* silencing also diminished *CCND1* mRNA expression in HepG2 cells. These results suggest that *CCND1* up-regulation contributes importantly to the mechanism of *URG4/URGCP*-mediated hepatocellular growth [14, 16–18].

In this preliminary study, the effects of RA treatment on *URG4/URGCP*, *CCND1*, *Bcl-2* and *Bax* gene expression changes in undifferentiated and differentiated SHSY5Y neuroblastoma cells was analyzed.

Material and methods

SH-SY5Y cell culture and differentiation

Human SH-SY5Y neuroblastoma cell line was grown in DMEM-Ham's F12 medium supplemented with 2 mM L-glutamine, penicillin (20 units/ml), streptomycin (20 µg/ml), and 10% (vol/vol) heat-inactivated fetal calf serum at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO_2. During culture, media were changed every 2 days and cells were replated before confluency. All experiments were conducted with exponentially growing cells. To induce differentiation, the cells were treated with 10 micromolar RA [19] in the dark for 3–10 days. SHSY5Y cells possess small processes in an undifferentiated state, and after treatment with RA, the cells developed long neurites, resembling a neuronal phenotype [20].

RNA extraction and semi-quantitative reverse transcription PCR

Total RNA was isolated with Tri-Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions with minor modifications and quantitated with a Nanodrop™ spectrophotometer (Thermo Scientific). RT reaction was performed using the First-Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol. Appropriate cycles were chosen to ensure the termination of PCR amplification before reaching a stable stage in each reaction. Gene expression was presented as the yield of PCR products from target sequences relative to the yield of PCR products from the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. PCR primers and reaction parameters are listed in Table 1. Semi-quantitative PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining and photographed under UV light. Gene expression was presented as the yield of PCR products from target sequences relative to the yield of PCR products from the *GAPDH* gene. In each instance, the amount of reverse transcription (RT)-PCR product for the gene of interest was normalized to the amount of *GAPDH* in the same sample. The experiments were repeated twice in duplicate in each group.

Results

The SH-SY5Y cells were initially treated with RA for 3–10 days. SHSY5Y cells possess small processes in an undifferentiated state, and after treatment with RA, the cells developed long neurites, resembling a neuronal phenotype. Treatment of SH-SY5Y cells with 10 micromolar RA in the culture medium resulted in neurite outgrowth that appeared on day 3 and extended on days 5, 7 and 10 (Fig. 1). This treatment yielded a nearly pure population of differentiated cells characterized by abundant neurite outgrowth. The cells were harvested on the 3rd, 5th, 7th and 10th days, and total RNA was extracted.

URG4/URGCP, *Cyclin D1*, *Bcl-2*, and *Bax* gene mRNA expression

The quality of RNA samples was confirmed by electrophoresis of RNA through 2% agarose gel stained with ethid-

Table 1. Primer sequences for quantitative reverse transcription (RT)-PCR

Primer name	Sequence
URG4/URGCP	F: 5'-CGGGAGATGGGACAGTTTTA-3'
URG4/URGCP	R: 5'-CATGGTGTTGAGGAGTGTGG-3'
Cyclin D1	F: 5'-AGCTCCTGTGCTGCGAAGTGGAAAC-3'
Cyclin D1	R: 5'-AGTGTTCAATGAAATCGTGCGGGGT-3'
Bcl-2	F: 5'-TTGGCCCCCGTTGCTT-3'
Bcl-2	R: 5'-CGGTTATCGTACCCCGTTCTC-3'
Bax	F: 5'-TCCCCCCGAGAGGTCTTTT-3'
Bax	R: 5'-CGGCCCCAGTTGAAGTTG-3'
GAPDH	F: 5'-CCCCACACACATGCACTTACC-3'
GAPDH	R: 5'-CCTAGTCCCAGGGCTTTGATT-3'

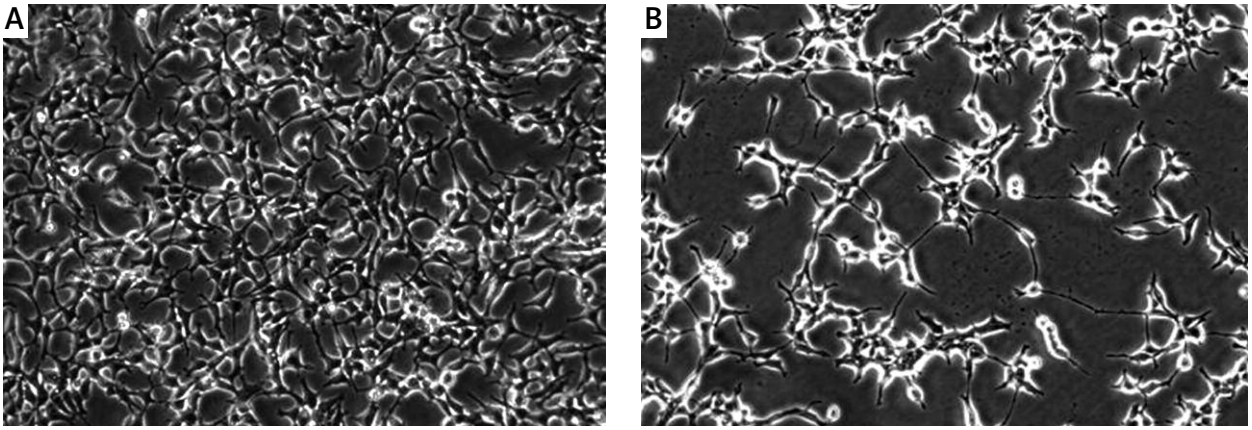
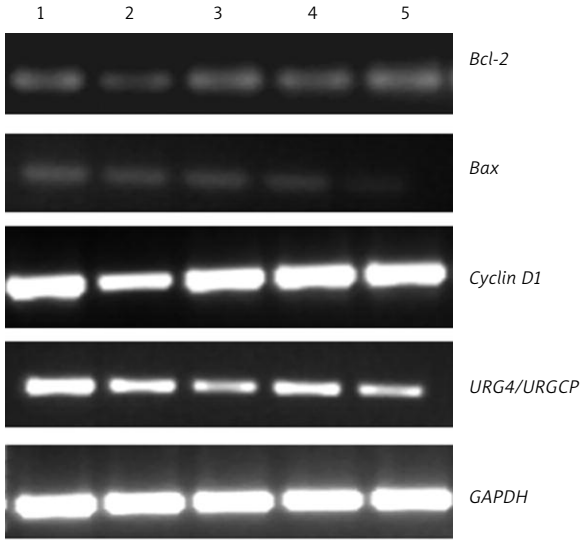


Fig. 1. Effect of RA on morphology of SHSY5Y cells. The cells were induced to differentiate for 10 days by adding 10 micromolar (final concentration) RA in medium containing 10% heat-inactivated fetal bovine serum. After every 24 h, medium was replaced with fresh medium containing 10 micromolar RA. **A)** Untreated cells (Olympus CKX41-X10); **B)** RA-treated cells (Olympus CKX41-X10)



1 – Control, 2 – RA-treated cells 3rd day, 3 – RA-treated cells 5th day, 4 – RA-treated cells 7th day, 5 – RA-treated cells 10th day

Fig. 2. Expression of *URG4/URGCP*, *CCND1*, *Bcl-2*, and *Bax* genes in SH-SY5Y cells

ium bromide. The A260/A280 ratio was between 1.9 and 2.0. The effect of RA on *URG4/URGCP*, *Cyclin D1*, *Bcl-2*, and *Bax* gene expression is shown in Fig. 2. Changes in mRNA levels, detected using semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR), were calculated as the proportion of the target gene amplification products to the amplification products of the housekeeping gene *GAPDH*. According to our results, *Bcl-2* and *CCND1* gene expression was increased in RA treated groups, while *URG4/URGCP* and *Bax* gene expression was decreased in SHSY5Y cells after RA treatment compared with the control cells (Fig. 2).

Discussion

Neuroblastoma (NB), which arises from cells of the neural crest, is the most common extra-cranial tumor of childhood. The behavior of neuroblastoma has puzzled investigators and is unique among malignant neoplasms. Defining the mechanisms responsible for its behavior may lead to the eventual successful treatment of this tumor. It is known that there has been increasing interest in recent years in the application of differentiation inducers in the therapy of NB. The genes and pathways that mediate the biological effects of RA have not been fully elucidated.

Retinoic acid is one of the antitumor agents that has been used successfully to treat certain human tumors including neuroblastomas [21]. Indeed, NB patients treated with RA have an increased survival rate without severe side effects [22]. Accumulating evidence suggests that RA plays an important role in the regulation of NB apoptosis as well as differentiation. However, certain neuroblastomas display an RA-resistant phenotype [23, 24]. 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.

Cell apoptosis is the process of programmed cell death that involves a series of biochemical events leading to a characteristic cell morphological change and death. Under physiological and pathological conditions, in the absence of such survival factors, cells enter a gene regulated program of self-elimination which depends on RNA and protein synthesis and on the expression of a specific set of genes. Apoptosis uses several genes related to the cell cycle and a very exciting point is the dependence of apoptosis on cell cycle phases. In our hands there is multiple cell cycle access to apoptosis. Various human NB cell lines express oncoproteins of the Bcl-2 family. These protein components of the apoptotic regulatory machinery control neuronal survival. Some of these components, such as *Bcl-2* and *Bcl-xL*, suppress apoptosis, while others, such as *Bax* and *Bak*, promote it [12, 25]. In mitochondrial pathways of apoptosis, Bcl-2 family proteins are critical determinants of mitochondrial membrane potential, which controls the cytoplasmic release of cytochrome c from mitochondria, thereby regulating apoptotic cell death. They are divided into two subfamilies based on their biological roles. The anti-apoptotic subfamily includes *Bcl-2* and *Bcl-xl* and the pro-apoptotic subfamily includes *Bax*, *Bim* and *Bmf*. The balance between these two groups determines the fate of cells. Anti-apoptotic Bcl-2 is one of the most important members that inhibit the mitochondria-dependent apoptotic pathway triggered by diverse cytotoxic agents through blocking mitochondrial permeability transition. Expres-

sion of pro- or anti-apoptotic proteins of the Bcl-2 family modifies the sensitivity to induced apoptosis. The overexpression of anti-apoptotic *Bcl-2* in neuronal cells was shown to prevent programmed cell death both *in vitro* and *in vivo* [26].

URG4/URGCP, a novel gene up-regulated in human hepatocellular carcinoma and gastric cancer, has been identified recently. *URG4/URGCP* is located on chromosome 7 (7p13). Previous data have suggested that *URG4/URGCP* might be an oncogene operating in hepatocarcinogenesis [14] and gastric carcinogenesis [16]. *URG4/URGCP* expression was found to be up-regulated in gastric cancer tissues compared with matched adjacent non-neoplastic tissues. *In vitro* observation showed that *URG4/URGCP* is up-regulated in gastric cancer cell lines compared with normal gastric epithelial cell lines, suggesting that *URG4/URGCP* might play an oncogenic role in the development of gastric cancer [16]. *CCND1* is a periodic regulatory protein that is believed to govern cell cycle transit from G1-phase into S-phase, and has been found to be abnormally expressed in many human cancers. Overexpression of *CCND1* leads to abnormal cellular proliferation, which underlies the process of tumorigenesis. Thus, *CCND1* can function as a cooperative oncogene in cell transformation. However, until now, there has been no investigation on *URG4/URGCP* expression in NB and also RA.

In our experiments, we investigated the influence of RA on a novel *URG4/URGCP* gene, and *CCND1*, *Bcl-2*, *Bax* gene expression changes in undifferentiated and differentiated SHSY5Y neuroblastoma cells were analyzed. The results showed that *Bcl-2* and *CCND1* gene expression was increased in RA treated groups from the 3rd to 10th days; and also *URG4/URGCP* and *Bax* gene expression was decreased in SHSY5Y cells after RA treatment compared with the control cells from the 3rd to 10th days. This study demonstrates the relationship between states of differentiation, and relative levels of *URG4/URGCP*, *CCND1*, *Bcl-2*, and *Bax* gene expression in neuroblastoma cells. To our knowledge, this is the first reported study of time-dependent changes in the gene expression of a human neuroblastoma cell line treated with RA. We suggest that RA may induce cell proliferation and escape apoptosis using a novel pathway by the *URG4/URGCP* gene. Further investigations are needed to clarify more direct transcriptional targets of RA signaling and the interaction of RA pathways with other pro-regenerative signals.

The authors declare no conflict of interest.

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Submitted: 18.09.2012
Accepted: 12.10.2012