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RESEARCH ARTICLE

Regulation of *URG4/URGCP* and *PPARα* gene expressions after retinoic acid treatment in neuroblastoma cells

Cigir Biray Avci · Yavuz Dodurga · Gulsah Gundogdu · Hasan Onur Caglar · Vural Kucukatay · Cumhur Gunduz · N. Lale Satiroglu-Tufan

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Abstract Neuroblastoma (NB), originating from neural crest cells, is the most common extracranial tumor of childhood. Retinoic acid (RA) which is the biological active form of vitamin A regulates differentiation of NB cells, and RA derivatives have been used for NB treatment. PPARα (peroxisome proliferator-activated receptor) plays an important role in the oxidation of fatty acids, carcinogenesis, and differentiation. *URG4/URGCP* gene is a proto-oncogene and that overexpression of *URG4/URGCP* is associated with metastasis and tumor recurrence in osteosarcoma. It has been known that *URG4/URGCP* gene is an overexpressed gene in hepatocellular carcinoma and gastric cancers. This study aims to detect gene expression patterns of *PPARα* and *URG4/URGCP* genes in SH-SY5Y NB cell line after RA treatment. Expressions levels of *PPARα* and *URG4/URGCP* genes were analyzed after RA treatment for reducing differentiation in SH-SY5Y NB cell line. To induce differentiation, the cells were treated with 10 μM RA in the dark for 3–10 days. Gene expression of *URG4/URGCP* and *PPARα* genes were presented as the yield of polymerase chain reaction (PCR) products from target genes compared with the yield of PCR products from the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. SH-SY5Y cells possess small processes in an undifferentiated state, and after treatment with RA, the cells developed long neurites, resembling a neuronal phenotype. *PPARα* gene expression increased in RA-treated groups; *URG4/URGCP* gene expression decreased in SH-SY5Y cells after RA treatment compared with that in the control cells. NB cell differentiation might associate with *PPARα* and *URG4/URGCP* gene expression profile after RA treatment.

Keywords *URG4/URGCP* · *PPARα* · Neuroblastoma cells · SH-SY5Y · Differentiation

C. B. Avci (✉) · C. Gunduz
Department of Medical Biology, School of Medicine, Ege University, Bornova, Izmir 35100, Turkey
e-mail: cbavci@gmail.com

Y. Dodurga
Department of Medical Biology, School of Medicine, Pamukkale University, Denizli 20020, Turkey

G. Gundogdu · V. Kucukatay
Department of Physiology, School of Medicine, Pamukkale University, Denizli 20020, Turkey

H. O. Caglar
Department of Stem Cell, Health Science Institute, Ege University, Bornova, Izmir 35100, Turkey

N. L. Satiroglu-Tufan
Department of Medical Genetics, School of Medicine, Pamukkale University, Denizli 20020, Turkey

Introduction

Neuroblastoma (NB), originating from neural crest cells, is the most common extracranial tumor of childhood. NB represents 8–10 % of all childhood tumors and is the most common reason of cancer-related deaths in infancy [1]. Cytogenetic studies have shown that different chromosomal rearrangements such as loss of 1p, loss of 11q, and gain of 17q are associated with NB prognosis and survival rates of patients. *MYCN* gene amplification and chromosomal rearrangements affect NB prognosis dependently with each other or independently [2]. Retinoic acid (RA) which is the biological active form of vitamin A has putative roles for developing organs and systems, including nervous system, in early embryonic period [3]. RA regulates the transition from the proliferating precursor cell to the post-mitotic differentiated cell and contributes to the morphological formation for distinct cell types [4–6]. RA derivatives have been used for NB treatment and improves the increasing survival rate of NB patients [7]. Peroxisome proliferator-activated

receptors (PPARs) are associated with inflammatory response, lipoprotein synthesis, and carcinogenesis [8–10]. Before transcriptional activation, PPARs heterodimerize with retinoid X receptor and complex binds to peroxisome response proliferator response element in the promoter region of target genes [11–13]. PPARα plays an important role in the oxidation of fatty acid, and PPARα regulates the expression of genes which are related to lipid and lipoprotein metabolism [14, 15]. It has been known that PPARα induces the development of cancer in the rodent liver and differentiation in epidermal keratinocytes [16, 17]. Increasing concentrations of all-trans-retinoic (atRA) acid or 9-cis retinoic acid (9cRA) induce brown adipocytes differentiation in culture condition [18]. When brown adipocyte cells are exposed to either atRA or 9cRA in a dose-dependent manner, PPARα mRNA expression increases in differentiating brown adipocytes [18]. Overexpression of *URG4/URGCP* gene which is located in 7p13 is associated with metastasis and tumor recurrence in osteosarcoma [19]. In metastasis, patients with high expression of *URG4/URGCP* exhibit shorter survival time [19]. It has been known that *URG4/URGCP* gene is an overexpressed gene in hepatocellular carcinoma, gastric cancer, and osteosarcoma [19–21]. High expression level of *URG4* in HepG2 cells promotes hepatocellular cancer cell growth and survival rate of cells in tissue culture and in nude mice [21]. In this study, expression levels of *PPARα* and *URG4/URGCP* genes were analyzed after RA treatment for reducing differentiation in SH-SY5Y NB cell line. This study aims to detect gene expression patterns of *PPARα* and *URG4/URGCP* genes in SH-SY5Y NB cell line after RA treatment.

Material and methods

SH-SY5Y cell culture and differentiation

Human SH-SY5Y neuroblastoma cell line was cultured in DMEM-Ham's F12 medium supplemented with 2 mM L-glutamine, penicillin (20 units/ml), streptomycin (20 μg/ml), and 10 % (*v*/*v*) 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 μM RA [22] in the dark for 3–10 days. SH-SY5Y cells possess small processes in an undifferentiated state, and after treatment with RA, the cells developed long neurites, resembling a neuronal phenotype.

RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated with Tri-reagent (Sigma, St. Louis, MO, USA) according to the manufacturer instructions and quantitated with a Nanodrop™ spectrophotometer (Thermo Scientific). Reverse transcription (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 genes compared with 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 were visualized by ethidium bromide staining and photographed under UV light. In each instance, the amount of RT-PCR product for the gene of interest was normalized to the amount of *GAPDH* in the same sample. The experiments were repeated two times using duplicates in each group.

Results

The SH-SY5Y cells were initially treated with RA for 3–10 days. SH-SY5Y 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 μM RA in the culture medium resulted in neurite outgrowth that appeared on day 3 and extended on days 5, 7, and 10. This treatment yielded a nearly pure population of differentiated cells characterized

Table 1 Primers used for RT-PCR

Primer name	Sequence	Amplicon size (bp)	Annealing (°C)
URG4	F: 5′-CGGGAGATGGGACAGTTTTA-3′ R: 5′-CATGGTGTTGAGGAGTGTGG-3′	285	55
PPARα	F : 5′-CTTCATCCTGAGTCCCTACCG-3′ R: 5′-GCCGTTCTGCTGCATTCG-3′	472	55
GAPDH	F: 5′-CCCCACACACATGCACTTACC-3′ R: 5′-CCTAGTCCCAGGGCTTTGATT-3′	98	55

by abundant neurite outgrowth. The cells were harvested on days 3, 5, 7, and 10, and total RNA was extracted.

URG4/URGCP and *PPARα* gene mRNA expression

The quality of RNA samples was confirmed by electrophoresis of RNA through a 2 % agarose gel stained with ethidium bromide. The A260/A280 ratio was between 1.9 and 2.0. The effect of RA on *URG4/URGCP* and *PPARα* gene expressions were shown in Figs. 1 and 2. Changes in mRNA levels, detected using semi-quantitative 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, *PPARα* gene expression increased in RA-treated groups; *URG4/URGCP* gene expression decreased in SH-SY5Y cells after RA treatment compared with that in the control cells (Figs. 1 and 2).

Discussion

Retinoids, like all-trans-retinoic acids, induces neuronal cell differentiation, and because of this effect, they have been used for NB therapy [7, 23]. Sung et al. designed three different differentiation processes which are microcell-mediated chromosome 1 transfer, *MYCN* gene knockdown, and 9-cis-retinoic acid treatment for IMR-32 NB cells, and expression levels of *STMN4* and *ROBO2* genes increased in IMR-32 cells for all differentiation method [24]. Besides upregulation of *STMN4* and *ROBO2* genes after differentiation of NB cells, RA can induce neuronal differentiation in neuroblastoma cells, which is associated with the induction of *HOX* genes [25, 26]. It has been known that RA treatment consistently induces cycle arrest of NB cells. RA increases G1 phase cells and decreases G2 phase cells in SK-N-SH cell line [27]. Qiao et al. demonstrated that expressions of CDK inhibitors p21 and p27, which are important in regulating the G1 phase checkpoint, are altered by RA in SK-N-SH cell line [27]. In addition to increased expressions of CDK inhibitors (p21 and p27), phosphorylated AKT and ERK1/2 expression levels are increased in time-dependent treatment with RA in SK-N-SH NB cells [27]. Qiao et. al also indicated that suppression of PI3K and ERK1/2 affects transcriptional activation of RA-RXR heterodimer in NB cells [27]. Although

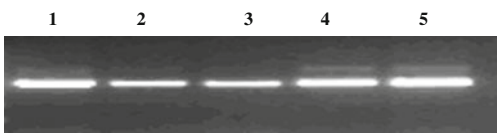

Fig. 1 Expression of peroxisome proliferator activation receptor (PPARα). RT-PCR analysis of PPAR showed increase of expression compared to that of the control, 3.25 % in 3 days (lane *2*), 8.94 % in 5 days (lane *3*), 7.72 % in 7 days (lane *4*), and 2.84 % in 10 days (lane *5*)

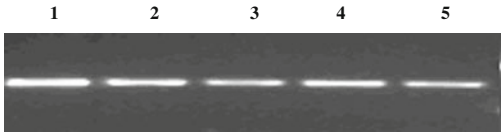

Fig. 2 Expression of upregulated gene 4 (URG4). RT-PCR analysis of URG4 showed decrease expression compared to that of the control 7.63 % in 3 days (lane *2*), 12 % in 5 days (lane *3*), 13.45 % in 7 days (lane *4*), and 28.72 % in 10 days (lane *5*)

signaling pathways and genes have been identified for NB cell differentiation after RA treatment, RA-induced neuronal differentiation has not been well understood. In this study, it was investigated whether expression profiles of *PPARα* and *URG4/URGCP* genes were associated with neuronal differentiation for SH-SY5Y NB cells after RA treatment in time-dependent manner. It has been known that *PPARα* induces the development of cancer in the rodent liver [28, 29] and *PPARα* activation, by its agonist WY14643, induces cell proliferation in MCF-7 and MDA-MB-231 breast cancer cell lines [30]. Although *PPARα* has oncogene role in hepatocellular carcinoma and breast cancer, *PPARα* activation induces cell cycle arrest and apoptosis in Ishikawa endometrial cancer cells [31]. Interestingly, *PPARα*-mediated differentiation process is activated by farnesol in epidermal keratinocytes [17]. In this study, SH-SY5Y neuroblastoma cell line was treated with 10 μM RA to induce neuronal differentiation [22]. Neurite outgrowth was observed after a 3-day period; afterwards, RA treatment and cell culture time were extended on days 5, 7, and 10 after the first RA treatment. We detected that SH-SY5Y neuroblastoma cell line expressed *PPARα* gene without RA treatment [32, 33], and *PPARα* expression was also detected in each time period for RA-mediated neural differentiation. *PPARα* expression after RA treatment was more than that of the control, not treated with RA, and *PPARα* expression increased after RA treatment in time-dependent manner, and there was a correlation between time-dependent doses of RA and *PPARα* expression. We showed that *PPARα* gene expression increases in RA-mediated neuronal differentiation. Contrary to *PPARα* expression, *URG4/URGCP* gene expression decreased in time-dependent manner after RA treatment when *URG4/URGCP* gene expression is compared to that of the control cells which were not treated with RA. Overexpression of *URG4/URGCP* gene have been detected in hepatocellular carcinoma, gastric cancer, and osteosarcoma [19–21]. siRNA-mediated suppression of *URG4/URGCP* gene in HEpG2 cell line and non-suppressed HEpG2 cell line exhibit different miRNA profiles. HEpG2 cell line which express *URG4/URGCP* gene shows alteration in miRNA patterns which are related with carcinogenesis [34]. Overexpression of *URG4/URGCP* gene reduces $p21^{Cip1}$ and $p27^{Kip1}$ cell-cycle inhibitors and upregulates cyclin D1 in hepatocellular carcinoma cells [35]. High expression of *URG4/URGCP*

gene in acute lymphoblastic leukemia patients is correlated with high expression of cell cycle related genes such as *CCNG1, CCNC,* and *CDC16* [36]. We detected that *URG4/URGCP* gene expression was decreased in SH-SY5Y cells after RA-treated cells compared to that in the control cells. In order that *URG4/URGCP* gene exhibits proto-oncogene function and induces cell cycle process, decreasing of *URG4/URGCP* gene might be important for differentiation of NB cells. NB cell differentiation might be associated with *PPARα* and *URG4/URGCP* genes expression profile after RA treatment.

Conflicts of interest None

References

1. Esiashvili N, Anderson C, Katzenstein HM. Neuroblastoma. Curr Probl Cancer. 2009;33:333–60.
2. Maris JM, Hogarty MD, Bagatell R, et al. Neuroblastoma. Lancet. 2007;369:2106–20.
3. Ross SA, McCaffery PJ, Drager UC, et al. Retinoids in embryonal development. Physiol Rev. 2000;80:1021–54.
4. Sidell N. Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells in vitro. J Natl Cancer Inst. 1982;68:589–96.
5. Andrews PW. Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. Dev Biol. 1984;103:285–93.
6. Kirschner SE, Ciaccia A, Ubels JL. The effect of retinoic acid on thymidine incorporation and morphology of corneal stromal fibroblasts. Curr Eye Res. 1990;9:1121–5.
7. Matthay KK, Reynolds CP, Seeger RC, et al. Long-term results for children with high-risk neuroblastoma treated on a randomized trial of myeloablative therapy followed by 13-cis-retinoic acid: a children's oncology group study. J Clin Oncol. 2009;27:1007–13.
8. Crisafulli C, Cuzzocrea S. The role of endogenous and exogenous ligands for the peroxisome proliferator-activated receptor alpha (PPAR-alpha) in the regulation of inflammation in macrophages. Shock. 2009;32:62–73.
9. Pyper SR, Viswakarma N, Yu S, et al. PPARalpha: energy combustion, hypolipidemia, inflammation and cancer. Nucl Recept Signal. 2010;8:e002.
10. Mukherjee R, Locke KT, Miao B, et al. Novel peroxisome proliferator-activated receptor alpha agonists lower low-density lipoprotein and triglycerides, raise high-density lipoprotein, and synergistically increase cholesterol excretion with a liver X receptor agonist. J Pharmacol Exp Ther. 2008;327:716–26.
11. Kliewer SA, Umesono K, Noonan DJ, et al. Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature. 1992;358:771–4.
12. Issemann I, Prince RA, Tugwood JD, et al. The retinoid X receptor enhances the function of the peroxisome proliferator activated receptor. Biochimie. 1993;75:251–6.
13. Miyata KS, McCaw SE, Marcus SL, et al. The peroxisome proliferator-activated receptor interacts with the retinoid X receptor in vivo. Gene. 1994;148:327–30.
14. Hashimoto T, Cook WS, Qi C, et al. Defect in peroxisome proliferator-activated receptor alpha-inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. J Biol Chem. 2000;275:28918–28.
15. Gross B, Staels B. PPAR agonists: multimodal drugs for the treatment of type-2 diabetes. Best Pract Res Clin Endocrinol Metab. 2007;21:687–710.
16. Roberts-Thomson SJ. Peroxisome proliferator-activated receptors in tumorigenesis: targets of tumour promotion and treatment. Immunol Cell Biol. 2000;78:436–41.
17. Hanley K, Komuves LG, et al. Farnesol stimulates differentiation in epidermal keratinocytes via PPARalpha. J Biol Chem. 2000;275:11484–91.
18. Valmaseda A, Carmona MC, Barbera MJ, et al. Opposite regulation of PPAR-α and -γ gene expression by both their ligands and retinoic acid in brown adipocytes. Mol Cell Endocrinol. 1999;154:101–9.
19. Huang J, Zhu B, Lu L, et al. The expression of novel gene URG4 in osteosarcoma: correlation with patients' prognosis. Pathology. 2009;41:149–54.
20. Song J, Xie H, Lian Z, et al. Enhanced cell survival of gastric cancer cells by a novel gene URG4. Neoplasia. 2006;8:995–1002.
21. Tufan NL, Lian Z, Liu J, et al. Hepatitis Bx antigen stimulates expression of a novel cellular gene, URG4, that promotes hepatocellular growth and survival. Neoplasia. 2002;4:355–68.
22. Sharma M, Sharma P, Pant HC. CDK-5-mediated neurofilament phosphorylation in SHSY5Y human neuroblastoma cells. J Neurochem. 1999;73:79–86.
23. Lopez-Carballo G, Moreno L, Masia S, et al. Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells. J Biol Chem. 2002;277:25297–304.
24. Sung PJ, Boulos N, Tilby MJ, et al. Identification and characterisation of STMN4 and ROBO2 gene involvement in neuroblastoma cell differentiation. Cancer Lett. 2012;328:168–75.
25. Manohar CF, Furtado MR, Salwen HR, et al. Hox gene expression in differentiating human neuroblastoma cells. Biochem Mol Biol Int. 1993;30:733–41.
26. Zha Y, Ding E, Yang L, et al. Functional dissection of HOXD cluster genes in regulation of neuroblastoma cell proliferation and differentiation. PLoS One. 2012;7:e40728.
27. Qiao J, Paul P, Lee S, et al. PI3K/AKT and ERK regulate retinoic acid-induced neuroblastoma cellular differentiation. Biochem Biophys Res Commun. 2012;424:421–6.
28. Peters JM, Cattley RC, Gonzalez FJ. Role of PPAR alpha in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator Wy-14,643. Carcinogenesis. 1997;18:2029–33.
29. Reddy JK, Rao MS, Azarnoff DL, et al. Mitogenic and carcinogenic effects of a hypolipidemic peroxisome proliferator, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14, 643), in rat and mouse liver. Cancer Res. 1979;39:152–61.
30. Suchanek KM, May FJ, Robinson JA, et al. Peroxisome proliferator-activated receptor alpha in the human breast cancer cell lines MCF-7 and MDA-MB-231. Mol Carcinog. 2002;34:165–71.
31. Saidi SA, Holland CM, Charnock-Jones DS, et al. In vitro and in vivo effects of the PPAR-alpha agonists fenofibrate and retinoic acid in endometrial cancer. Mol Cancer. 2006;5:13.
32. Isaac AO, Kawikova I, Bothwell AL, et al. Manganese treatment modulates the expression of peroxisome proliferator-activated receptors in astrocytoma and neuroblastoma cells. Neurochem Res. 2006;31:1305–16.
33. Tippmann F, Hundt J, et al. Up-regulation of the alpha-secretase ADAM10 by retinoic acid receptors and acitretin. FASEB J. 2009;23(6):1643–54.

34. Dodurga Y, Yonguc GN, Avci CB, et al. Investigation of microRNA expression changes in HepG2 cell line in presence of URG4/URGCP and in absence of URG4/URGCP suppressed by RNA interference. Mol Biol Rep. 2012;39:11119–24.
35. Xie C, Song LB, Wu JH, et al. Upregulator of cell proliferation predicts poor prognosis in hepatocellular carcinoma and contributes to hepatocarcinogenesis by downregulating FOXO3a. PLoS One. 2012;7:e40607.
36. Dodurga Y, Oymak Y, Gunduz C, et al. Leukemogenesis as a new approach to investigate the correlation between up regulated gene 4/upregulator of cell proliferation (URG4/URGCP) and signal transduction genes in leukemia. Mol Biol Rep. 2013;40:3043–8.