Inhibition of all-trans retinoic acid on *MDM2* gene expression in astrocytoma cell line SHG-44

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Abstract: Objective To investigate the impact of all-trans retinoic acid (ATRA) on *MDM2* gene expression in astrocytoma cell line SHG-44, and to provide basic data for further research on the progression mechanism and gene therapy of human astrocytoma. **Methods** The differential expressions of *MDM2* gene and protein in SHG-44 cells were detected by cDNA microarray and Western blot, respectively, before and after treatment of ATRA. The expressions of *MDM2* protein in WHO grade II and grade IV astrocytomas were determined by immunohistochemical streptavidin-peroxidase method. Some differentially expressed genes were selected randomly for Northern blot analysis. **Results** The intensity ratio of ATRA-treated to untreated SHG-44 cell was 0.37 in the cDNA microarray, suggesting that the expression of *MDM2* gene was down-regulated in SHG-44 cells after treatment with ATRA. Some genes differentially expressed in the microarray were confirmed by Northern blot. Western blot demonstrated that the optical density ratios of *MDM2* to β -actin in ATRA-treated and untreated SHG-44 were 14.02 ± 0.35 and 21.40 ± 0.58 ($t = 24.728$, $P = 0.000$), respectively, suggesting that the expression of *MDM2* protein was inhibited in ATRA-treated SHG-44 cells. Moreover, the percentages of *MDM2*-positive protein were 24.00% (6/25) and 56.52% (13/23) ($\chi^2 = 5.298$, $P = 0.021$) in WHO grade II and grade IV astrocytomas, respectively, suggesting that the expression of *MDM2* protein may increase along with the elevation of astrocytoma malignancy. **Conclusion** ATRA can inhibit *MDM2* gene expression in SHG-44 cells, and *MDM2* is related to astrocytoma progression.

Keywords: all-trans retinoic acid; astrocytoma; SHG-44 cell line; *MDM2*; cDNA microarray

1 Introduction

Astrocytoma, the most common type of brain tumors, could be classified into four grades based on tumor cell differentiation, proliferation, and so on. Pilocytic astrocytoma (WHO grade I) and low-grade diffuse astrocytoma (WHO grade II) are well-differentiated tumors that usually develop in young patients. These tumors grow relatively slow but have the potential to progress to anaplastic astrocytoma

(WHO grade III) and may also transform into glioblastoma multiforme (WHO grade IV)^[1]. Malignant astrocytomas are difficult to be resected thoroughly because of their invasive properties, and the brain structures critical to proper cognitive, behavioral, and motor functions are likely to be damaged during the operation. Targeted therapeutic approaches based on the knowledge of biological progression could improve the prognosis of astrocytoma. On the other hand, differentiation induction as a treatment for malignant tumor is an attractive topic in researches on cancer therapy. Some specific drugs, such as all-trans retinoic acid (ATRA), could induce differentiation of high-grade tumors and make tumor cells become less malignant or even normal. In this study, we treated astrocytoma cell line SHG-44 with ATRA and detected the differential expression of *MDM2* gene in ATRA-treated and untreated

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SHG-44 cells by cDNA microarray to explore the relationship between *MDM2* gene expression and astrocytoma progression.

2 Materials and methods

2.1 Cell culture and reagents SHG-44 cell line, established by Du^[2], was kindly provided by Prof. Xiu-Wu BIAN (Department of Pathology, the Third Military Medical University, Chongqing, China). According to Du's report, SHG-44 cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, and added with 3% *L*-glutamine (10 μ L/mL), 100 IU/mL penicillin, and 100 μ g/mL streptomycin, at 37 °C in a humidified atmosphere with 5% CO₂ in the air. The media was changed 2-3 times weekly and at 24 h before SHG-44 cells were collected. All the media and reagents used in the cell culture were purchased from Gibco Company (Gibco-BRL, New York, USA).

2.2 Treatment of SHG-44 cells with ATRA According to several recent reports on the effects of ATRA on glioma cell lines and astrocytoma tissues^[3], we adopted a widely-used ATRA concentration at 10 μ mol/L^[4] for 7 d in our experiment. ATRA (Sigma, St. Louis, MO, USA) was dissolved in the culture media described above and diluted to the concentration of 10 μ mol/L for cell culture. SHG-44 cells were cultured in the foregoing treated culture media that contained 10 μ mol/L ATRA.

2.3 Detection of cell proliferation by cell-counting assay SHG-44 cells (1 $\times$ 10⁵/mL) were plated on 6-well plates (1.0 mL each well), divided into ATRA-treated and untreated group, and each group consisted of 3 wells. The media were changed 2-3 times every week. Cells were collected by trypsinization (trypsin-EDTA, 1 $\times$; Gibco-BRL) every day until day 7, and counted by using a Hemacytometer.

2.4 Analysis of cell cycle using flow cytometry Cell cycle was analyzed by flow cytometry. Briefly, SHG-44 cells were harvested by trypsinization and adjusted to a density of about 5 $\times$ 10⁵ cells/mL. The cells fixed in 75% ethanol/PBS supplemented with 1% fetal bovine serum, and then washed with PBS for 3 times, followed by staining with 20 μ g/mL of propidium iodide (Sigma, St. Louis, MO, USA). After treated with 10 μ g/mL DNase-free RNase (Boehringer Mannheim, Germany) for 1 h, the samples were analyzed by using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA).

2.5 Extraction of total RNA After treatment with ATRA, total RNA of SHG-44 cells was extracted with Trizol Reagents

(Gibco BRL) according to the manufacturer's instruction. Briefly, about 1 $\times$ 10⁷ cells were collected in 4 mL of denaturing solution. After two phenol/chloroform extractions, RNA was precipitated with isopropanol, washed with 75% ethanol and air-dried. The extracted total RNA was re-suspended in the RNase-free solution (Ambion, Austin, Texas, USA) and stored at -20 °C. The concentration of total RNA was measured with Biophotometer (Eppendorf, Hamburg, Germany). The quality of RNA was verified by agarose gel electrophoresis.

2.6 Detection of gene expression profiles by cDNA microarray The cDNA microarray that contains 9 182 known genes was prepared and provided by Dr. Zhong YANG (the Third Military Medical University, Chongqing, China). After reverse transcription of 150 μ g total RNA with Superscript II reverse transcriptase (Gibco-BRL), the cDNA products of ATRA-treated and untreated SHG-44 were obtained and labeled with the fluorescent dyes Cy5 and Cy3, respectively. Then, hybridization was carried out at 65 °C overnight. The microarray image was scanned by GenePix 4000B and analyzed by GenePix Pro 3.0 software (Axon, Union City, CA, USA).

A differentially expressed gene was confirmed by the following criteria: (1) the gene appeared at least two times of the triplicate microarray hybridizations, and the normalized intensity ratio of background subtracted Cy5 to Cy3 (Cy5/Cy3) was greater than 2.0 (up-regulated) or less than 0.5 (down-regulated); (2) the Cy5/Cy3 value in the third microarray had no contrary trend.

2.7 Northern blot The differentially expressed genes were verified by Northern blot according to manufacturer's instruction. A few of cDNA clones serving as probes for Northern blot were selected randomly and individually amplified by PCR to confirm the results of microarray. Beta-actin was used as an internal control.

2.8 Western blot analysis MDM2 protein (30 μ g) was prepared according to the manufacturer's instruction and detected by Western blotting. The anti-MDM2 monoclonal antibody (1:1 000; Santa Cruz, CA, USA) was incubated with the MDM2 proteins transferred on the Immobilon P membrane (Millipore) for 12 h at 4 °C. The immunoreactive proteins were incubated with a goat anti-mouse immunoglobulin conjugated to horseradish peroxidase, followed by chemoluminescence detection for visualization. The β -actin protein was used as an internal control. The expression level of MDM2 was presented by the optical density (OD) ratio of MDM2/ β -actin.

2.9 Immunohistochemistry (IHC) detection in astrocytomas specimens

The streptavidin-peroxidase method was adopted to detect the expressions of MDM2 proteins in 48 astrocytomas surgical specimens, which were collected from West China Hospital, Sichuan University, China. All specimens were divided into grade II ($n = 25$) and grade IV astrocytomas ($n = 23$) according to their atypia, mitosis, vascular angiogenesis, and necrosis. Briefly, the 4- μm slides were processed for antigen retrieval using microwave heating in citrate buffer (10 mmol/L, pH 6.0) for 20 min followed by cooling to room temperature and then immunostaining with commercially available antibodies according to the manufacturer's recommendations. The human monoclonal anti-MDM2 antibody (Maixin-Bio, Fuzhou, China) was diluted to 1:300 in primary antibody diluent, and the SP-9000 Histostain-Plus Kits was purchased from ZSGB-BIO (Beijing, China). The percentage of IHC-positive cells was scored by a blinded investigator under light microscope, and the positive criteria was set at more than 5% positive cells^[5]. Digital images were captured using an Olympus BX60 microscope equipped with a digital DVC video camera. The known positive slides were used as positive controls, while negative controls were obtained by replacing the primary antibody with PBS.

2.10 Statistical analysis Data were analyzed by *t*-test, Pearson χ^2 test, and Spearman analysis with SPSS 13.0 for Windows. Each experiment was repeated for three times. Results are presented as mean $\pm$ SD. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Morphology and proliferation of SHG-44 cells after treatment of ATRA

ATRA induced differentiation and inhibited proliferation of SHG-44 cells. Untreated SHG-44 cells were ellipse, with little cytoplasm, big nuclei, increased karyoplasm proportion, and few short prominences. However, ATRA-treated SHG-44 cells were short shuttle in shape, with small nuclei, decreased karyoplasm proportion, and increased thin cytoplasmic processes (Fig. 1). Furthermore, the growth curves of ATRA-treated and untreated SHG-44 cells indicated that the proliferation speed of ATRA-treated SHG-44 cells was lower than that of untreated SHG-44 cells ($P < 0.05$; Fig. 2, Table 1).

3.2 Cell cycle of SHG-44 cells after treatment of ATRA

ATRA inhibited growth of SHG-44 cells, but did not induce cell apoptosis. On Day 3, the cell proportions at G0/G1, S,

Fig. 1 Cell morphology of ATRA-treated and untreated SHG-44 cells under light microscope (Hematoxylin-Eosin staining). A: ATRA-treated cells were short shuttle in shape, with small nuclei, decreased karyoplasm proportion, and increased thin cytoplasmic processes. B: Untreated cells were ellipse, with little cytoplasm, big nuclei, increased karyoplasm proportion, and few short prominences. Scale bar, 100 μm .

Fig. 2 Growth curves of SHG-44 cells with and without treatment of ATRA. The 7-d growth curves indicated that the rate of cell proliferation was decreased after treatment with ATRA.

Fig. 3 Overlapped hybridization image and scatter plot in cDNA microarray. A: The overlapped hybridization image of SHG-44 cells with and without 3-d treatment of 10 μ mol/L ATRA. B: The scatter plot of the normalized log10 intensity of the background subtracted Cy5 (ATRA-treated SHG-44 cells) versus Cy3 (untreated SHG-44 cells). The genes located outside of the dot lines are significantly differentially expressed by at least 2 folds.

Fig. 4 Comparison between the results of Northern blot (A) and corresponding cDNA microarray (B). The expression of β -actin was used for internal control. Northern blot showed that the expression changes of ribosomal protein L13 (down-regulation) and superoxide dismutase 2 (up-regulation) had the same trends to those in the cDNA microarray.

Table 1 Number of the SHG-44 cells with or without ATRA treatment

Time	Cell count* ($\times 10^5/\text{mL}$)		<i>P</i> value
	Untreated SHG-44	ATRA-treated SHG-44	
Day 1	1.9 $\pm$ 0.20	1.6 $\pm$ 0.10	0.081
Day 2	6.3 $\pm$ 0.26	4.9 $\pm$ 0.20	0.002
Day 3	12.3 $\pm$ 0.36	8.1 $\pm$ 0.17	< 0.001
Day 4	20.4 $\pm$ 0.50	10.7 $\pm$ 0.30	< 0.001
Day 5	37.4 $\pm$ 0.56	21.7 $\pm$ 0.44	< 0.001
Day 6	38.2 $\pm$ 0.46	23.7 $\pm$ 0.40	< 0.001
Day 7	38.1 $\pm$ 0.53	25.6 $\pm$ 0.36	< 0.001

*All values are presented as mean $\pm$ SD of 3 wells in each group

and G2/M phases were (76.9 $\pm$ 6.4)%, (13.8 $\pm$ 1.7)%, and (9.3 $\pm$ 0.9)%, respectively, in ATRA-treated SHG-44 cells, and were (61.9 $\pm$ 5.1)%, (25.3 $\pm$ 1.9)%, and (12.8 $\pm$ 1.3)%, respectively, in untreated SHG-44 cells. Cell cycle was significantly arrested at G0/G1 phase after treatment of ATRA ($P < 0.05$).

3.3 Differentially expressed genes in SHG-44 cells after treatment of ATRA Figure 3 shows an overlapped hybridization image of Cy5 (ATRA-treated SHG-44) and Cy3 (untreated SHG-44), as well as a scatter plot of the normalized log10 intensity of the background subtracted Cy5 versus Cy3. Total 42 differentially expressed genes were identified. Among them, 28 genes were up-regulated and 14 were down-regulated in ATRA-treated SHG-44 cells compared with those in untreated SHG-44 cells. These genes were involved in apoptosis, cell mobility and metastasis, cell cycle and growth regulation, cytoskeleton, differentiation, metabolic pathway, oncogene, oxidative phosphorylation, receptors and signal transduction, ribosome, ubiquitin-proteasome system, growth factor and cytokine, and so on. Those differentially expressed

genes related to apoptosis and growth regulation were listed in Table 2.

Northern blot results showed the same trends of down-regulation of ribosomal protein L13 gene (*RPL13*) and up-regulation of superoxide dismutase 2 gene (*SOD2*) determined by cDNA microarray (Fig. 4).

3.4 Western blot analysis In this investigation, Western blot demonstrated that the OD ratios of MDM2/ β -actin were 14.02 $\pm$ 0.35 and 21.40 $\pm$ 0.58 ($t = 24.728$, $P = 0.000$), respectively, in ATRA-treated and untreated SHG-44 cells. MDM2 protein

Fig. 5 Western blot analysis of MDM2 in SHG-44 cells. MDM2 protein expressed differentially between untreated SHG-44 cells (lane 1) and ATRA-treated SHG-44 cells (lane 2). The histogram shows the relative optical density (OD) of each band by referring the amount of β -actin. The expression level of MDM2 protein in Western blot was in accordance with that of MDM2 detected by cDNA microarray at RNA level.

Table 2 Differentially expressed genes related to apoptosis and growth regulation

Unigene No.	Accession	Gene symbol	Gene description	Ratio*
Apoptosis				
Hs.166468	AA452724	<i>PDCD5</i>	programmed cell death 5	4.82
Hs.318885	BG035651	<i>SOD2</i>	superoxide dismutase 2, mitochondrial	2.26
Hs.86131	AA43075	<i>FADD</i>	Fas (TNFRSF6)-associated via death domain	2.03
Cell cycle & growth regulation				
Hs.84153	BI090082	<i>DCTN2</i>	dynactin 2 (p50)	2.00
Hs.170027	NM_006878	<i>MDM2</i>	Mdm2 p53 binding protein homolog (mouse)	0.37
Growth factor & cytokine				
Hs.197540	AU134078	<i>HIF1A</i>	hypoxia-inducible factor 1, α subunit	3.99

*Ratio = ATRA-treated SHG-44/untreated SHG-44 (Cy5/Cy3)

Fig. 6 Expressions of MDM2 in the nucleus of grade II (A) and grade IV (B) gliomas (Streptavidin-Peroxidase staining). The MDM2-positive cells were more obviously in grade IV gliomas, suggesting that the expression of MDM2 protein is associated with the elevation of astrocytoma malignancy. Scale bar, 100 μ m.

expression in untreated SHG-44 cells was higher than that of 10 μ mol/L ATRA-treated SHG-44 cells, suggesting that the expression of MDM2 protein was inhibited in SHG-44 cells after treatment with ATRA. The expression level of MDM2 protein in Western blot was in concord with that of *MDM2* at RNA level as detected by cDNA microarray. Figure 5 shows the images and quantitative analysis of MDM2 protein by Western blotting.

3.5 Analysis of IHC in astrocytoma specimens The MDM2-positive protein was located in the nucleus (Fig. 6), and the positive rates of MDM2 protein were 24.00% (6/25) and 56.52% (13/23) ($\chi^2 = 5.298$, $P = 0.021$), respectively, in WHO grade II and grade IV astrocytomas. Spearman rank analysis demonstrated that there was a positive correlation between the expression of MDM2 and the malignancy of astrocytomas. The results suggested that the expression of MDM2 protein increased in astrocytomas along with the elevation of astrocytoma malignancy.

4 Discussion

Apart from the differentiation-promoting effect, ATRA also inhibits cell proliferation and induces cell cycle arrest^[6]. In our experiments, this is also the case. In addition, the gene expression profile of SHG-44 cells was observed changing after treatment with ATRA, and 42 differentially expressed genes were accordingly identified. Among them, 28 were up-regulated and 14 were down-regulated in ATRA-treated SHG-44 cells as compared with the untreated SHG-44 cells. These genes are involved in various aspects of cell biology, such as apoptosis, cell mobility and metastasis, cell cycle

and growth regulation, and so on. The stable up-regulation of Fas-associated via death domain (*FADD*), programmed cell death 5 (*PDCD5*), superoxide dismutase 2, mitochondrial (*SOD2*) and hypoxia-inducible factor 1, α subunit (*HIF1* α), as well as repression of *Mdm2* p53 binding protein homolog (mouse) (*MDM2*), were observed in ATRA-treated SHG-44 cells. Western blotting demonstrated that the expression level of MDM2 protein was consistent with that of *MDM2* at RNA level detected by DNA microarray. In this context, the relationship between *MDM2* and tumor evolution would be explained as follows.

MDM2 locus is located on chromosome 12q13-14, and encodes a 483-amino acid residue MDM2 protein. Biochemically, MDM2 functions as an E3 ubiquitin ligase responsible for ubiquitination and degradation of p53. MDM2 protein contains the following 5 domains: the p53 interaction domain, the nuclear localization signal and nuclear export signal, the central acidic domain, the zinc finger domain, and the RING domain^[7]. In normal cells, the primary function of MDM2 is to inhibit p53 activity and participates in many of the cellular physiological processes, such as cell cycle and growth regulation, differentiation, transcription, and so on^[8].

MDM2 is a primary negative regulator of p53. It can inhibit p53 functions as a transcription factor and induce p53 degradation by initiating the ubiquitination and nuclear export of p53^[9]. Interestingly, *MDM2* itself is a target for direct transcriptional activation by p53, leading to increased MDM2 protein. This negative feedback loop is essential for controlled activation of p53 in cells that have limited damage and can self-repair. It was reported that overexpression of *MDM2*

might lead to insufficient p53 activity and contributes to tumorigenesis^[10], but inhibition of *MDM2* could induce apoptosis and suppress proliferation^[11]. The *MDM2* oncogene is overexpressed in many human malignancies, including in gliomas^[8,11]. Slack *et al.*^[8] reported that the increased constitutive transcriptional activation of *MDM2* might contribute to oncogenesis and neuroblastoma development. In the present study, the results showed that in the SHG-44 cells, the down-regulation of *MDM2* caused by ATRA treatment was accompanied by inhibition of cell proliferation and cell cycle arrest at G0/G1 phase, suggesting that the up-regulation of *MDM2* might contribute to the progression of astrocytomas.

In this study, IHC analysis showed that MDM2 protein was overexpressed in grade IV astrocytomas, and its level was positively related to the histopathological grade of astrocytomas. MDM2 overexpression has been documented in gliomas, and the increase of MDM2 protein can attenuate the p53 function, which can lead to cancer formation and progression of gliomas^[12,13]. These results implied that high MDM2 levels were associated with poor clinical prognosis^[11]. Our results showed that MDM2 expression was closely associated with the malignant degree of astrocytomas, suggesting that MDM2 overexpression might contribute to tumorigenesis and progression of astrocytoma. Thus MDM2 could be a relevant marker in determining the prognosis of patients with astrocytomas and could provide a more objective way to classify astrocytomas^[14]. Respecting the role of MDM2 in the progression of astrocytomas, previous report showed that Nutlin 3a, an inhibitor of MDM2 protein, can cause growth inhibition of neuroblastoma cells^[15]. Moreover, Michiue *et al.*^[16] reported that suppression of MDM2-mediated ubiquitination by a specific mutant protein, which effectively delivered in glioma cells, can inhibit the proliferation of glioma cells. Therefore, the p53 regulatory gene *MDM2* might be an effective therapeutic target in the future.

In summary, ATRA treatment can inhibit *MDM2* gene expression in SHG-44 cells. The expression of *MDM2* is closely associated with the malignant degree of the astrocytomas, and can be used as a valuable indicator for deciding prognosis of astrocytomas.

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全反式维甲酸抑制 *MDM2* 基因在胶质瘤细胞 SHG-44 中的表达

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摘要: **目的** 探讨全反式维甲酸对胶质瘤细胞 SHG-44 中 *MDM2* 基因表达的影响, 为进一步研究脑胶质瘤的进展机制及基因治疗提供依据。**方法** 分别利用 cDNA 微阵列与 Western blot 技术分析在 10 $\mu\text{mol/L}$ 全反式维甲酸(all-trans retinoic acid, ATRA)处理前后的胶质瘤 SHG-44 细胞中 *MDM2* 基因和蛋白的差异表达; 应用免疫组化链霉菌抗生物素蛋白-过氧化酶(Streptavidin-Peroxidase, SP)法检测 II 级与 IV 级胶质瘤标本 *MDM2* 蛋白的表达。随机选择数个差异基因进行 Northern 杂交实验, 以验证 cDNA 微阵列的结果。**结果** 应用 cDNA 微阵列检测发现, *MDM2* 基因在 ATRA 处理与未处理的 SHG-44 细胞之间表达量的比值为 0.37, 提示 ATRA 可抑制 *MDM2* 基因在 SHG-44 中的表达。该结果进一步得到 Northern 杂交实验结果的支持。Western blot 分析结果显示 10 $\mu\text{mol/L}$ ATRA 处理前后胶质瘤 SHG-44 细胞之间 *MDM2* 蛋白的相对表达量分别为 21.40 ± 0.58 和 14.02 ± 0.35 ($t = 24.728$, $P = 0.000$), 提示 *MDM2* 蛋白在 SHG-44 中的表达受到 ATRA 抑制。II 级和 IV 级胶质瘤标本 *MDM2* 蛋白的阳性表达率分别为 24.00%(6/25)和 56.52%(13/23) ($\chi^2 = 5.298$, $P = 0.021$), *MDM2* 蛋白的表达随胶质瘤恶性程度的增高而增加。**结论** ATRA 可抑制 SHG-44 胶质瘤细胞中 *MDM2* 基因的表达, *MDM2* 基因的表达水平与胶质瘤的演进有关。

关键词: 全反式维甲酸; 胶质瘤; SHG-44 细胞系; *MDM2*; cDNA 微阵列