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Influence of all-trans retinoic acid(ATRA) on the expression of NANOG in Glioma cell lines

Liu Weixian, *Yi Fuxin*△

(*Department of Neurosurgery*, *the First Affiliated Hospital*, *Liaoning Medical University*, *Jinzhou*, *Liaoning* 121001, *China*)

Abstract: **Objective** To investigate the effects of all-trans retinoic acid(ATRA) on the expression of NANOG in glioma cell lines. **Methods** Each cell line was divided into the experimental group which was treated with ATRA for 5 days, and the control group which was cultured normally without ATRA treatment. Immunocytochemistry and RT-PCR were adopted to detect the expression of NANOG at protein and mRNA level among the three kinds of cell lines. **Results** Positive rates of NANOG protein in glioma cell lines SHG-44, U87 MG and U251 in control groups were (65.5±3.0)%, (64.8±8.0)% and (64.5±1.2)%, respectively, and the difference was not statistically significant($F=0.190$, $P=0.829$). *NANOG* mRNA of the three cell lines in the relative content were 0.636 8±0.039 9, 0.642 1±0.063 7, 0.651 6±0.044 4, and the difference was not statistically significant($F=0.427$, $P=0.662$). However, 5 days after application of ATRA-induced NANOG protein in the three cell lines, the positive rates of NANOG protein of experimental groups were (36.5±7.3)%, (35.5±7.9)%, (35.2±6.1)%, respectively, compared with the control groups, the differences were statistically significant ($F_{SHG-44}=259.1$, $F_{U87}=129.5$, $F_{U251}=431.8$, $P_{SHG-44}=0.0$, $P_{U87}=0.0$, $P_{U251}=0.0$), and the relative level of *NANOG* mRNA in these groups were 0.458 3±0.079 1, 0.255 1±0.079 3 and 0.333 1±0.054 0, respectively, compared with the control groups, the difference was significant($F_{SHG-44}=77.8$, $F_{U87}=277.9$, $F_{U251}=398.1$, $P_{SHG-44}=0.0$, $P_{U87}=0.0$, $P_{U251}=0.0$). **Conclusion** NANOG which highly expressed in glioma cell line SHG-44, U87 MG and U251 can be reduced by ATRA.

Key words: retinoic acid; glioma; reverse transcriptase-polymerase chain reaction; immunocytochemistry; NANOG protein

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Glioma, with a high incidence and relapse after surgery, is originated in neural epiblast , and the most common malignant tumor of the central nervous system, which has a high incidence, and is non-sensitive to radiotherapy and chemotherapy, so the characteristics of the patient's mortality rate is quite high. In the treatment of gliomas in clinical, surgery is given priority to it, because the tumor with no specific demarcation of brain tissue, shows an invasive growth, which is difficult to removed completely. Therefore, postoperative radiotherapy, chemotherapy and immunotherapy are essential. Despite comprehensive treatment adopted for glioma, after 5 years of survival rate is still low, it is difficult to cure completely.

In recent years, cancer stem cells as the source of the deepening of theoretical research, it is increasingly recognized that biological characteristics of tumor including tumor origin, tumorigenicity, malignancy, drug resistance, recurrence and metastasis are related to certain stem cell genes which are abnormally expressed. In 2003, NANOG, which is an important facor, was newly discovered in embryonic stem cells by Mitsui K et al.[1] and Chambers et al.[2]. NANOG as the maker of totipotency or pluripotency of stem cell plays an important role for maintaining cell self-renewal, proliferation and totipotency and becomes a focus in recent years. With further research, NANOG, in some germ cell tumors, such as testicular carcinoma in situ, seminoma[3-4] and embryonal carcinoma cell line NCCIT[5], as well as somatic cancers like breast cancer, bladder cancer, cervical cancer[6], colon cancer[7], prostate cancer, liver cancer[8-10], oral squamous cell carcinoma, gastric cancer[11] have been reported to be expressed , which are, in some degree, considered to be related to treatment resistance, recurrence, metastasis, malignancy of cancer.

Induced-differentiation therapy is a new field of cancer chemotherapy. With the thorough study of tumor molecular biology, it has been already proved that the original embryonic phenotype of cancer cells (including glioma cells) can be reversed to normalities by differetiation of reduction cancer cells[12], although there are genetic abnormalities. Induced-differentiation therapy is based on the above understanding, that is, the application of chemical drug could induced the differentiation and reverse the proliferation, invasion, metastasis and other malignant phenotype, making it normal or near normal cells(tumor differentiation or cancer reversed), so as to cure cancer. With the deepening of research, in particular, the successful application of induction of differentiation therapy in acute promyelocytic leukemia, neuroblastoma and other tumors in the successful application, people's interest has been stimulated by the therapy's application in solid tumors, and some academics have already begun to explore the therapy in the treat-

△ **Corresponding author**, Tel:15940676888; E-mail:leotongtong@163.com。

ment of the potential use of glioma[13]. All-trans retinoic acid (ATRA) is the most important one in a variety of retinoic acid isomers, which is researched most thoroughly, most applicated in clinical, with the strongest differentiation-inducing role. It can induce, including prostate cancer, leukemia, stomach cancer, liver cancer, glioma and other tumor cell , differentiation and apoptosis, and inhibit the proliferation of these cells.

Since ATRA can induce tumor cells, first we hypothesize that ATRA can induce the expression of NANOG reduced, leading to the differentiation of glioma cells. According to several recent reports on the effects of ATRA on glioma cell lines and astrocytoma tissue, we adopted a widely-used ATRA concentration at 10 μmol/L in our experiment to induce human-derived glioma cell lines SHG-44, U87 MG, U251 for 5 days for exploring the effects of ATRA on the expression of NANOG, and laying the fundation for further research of NANOG in the resistance, recurrence, metastasis of glioma.

1 Materials and methods

1.1 Major reagents and instruments DMEM was obtained from Gibco(USA). ATRA was purchased from Sigma(USA). Fetal bovine serum(FBS) was purchased from Hyclone(USA). Antibody (NANOG) was purchased from Bioss(USA). Trizol and reverse transcriptase-polymerase chain reaction(RT-PCR) kit were purchased from TaKaRa(Dalian, China). PCR Primers were synthesized by Shanghai Sangon Biotechnology Co. Ltd. Major instruments included BB16 CO_2 incubator(Heraeus and Lishen company, Germany), inverted phase contrast microscope (Olympus, Japan) and RT-PCR instrument(ABI, USA).

1.2 Cell culture and induction Glioma cell lines U87 MG, U251, SHG-44 used in the experiment were all purchased from Shanghai Cell Bank of the Chinese Academy of Science, cultured in Dulbecco's modified Eagle medium(DMEM) with 10% heat-inactivated FBS, penicillin and streptomycin cocktail. All cells were cultured in a humidified incubator at 37℃, 5% CO_2. The media was changed 1－2 times 24 hours before cells were passaged.

1.3 Detecting NANOG expression by immunocytochemistry Cells in control groups were cultured normally and those in experimental groups were treated with ATRA for 5 days, which were re-suspended in culture medium containing 10% FBS, and dropped onto poly-L-lysine-coated coverslips. After standing still for about 4 hours until the solution adhered to the coverslips, these coverslips were fixed in 4% paraformaldehyde for at least 30 min at room temperature, treated cells with 3% H_2O_2 and pure methanol 1 to 50 for 30 min, permeabilized with 0.3% Triton X-100 for 30 min, then blocked with bull serum albumin (BSA) for at leat 30 min, incubated with rabbit anti-human NANOG antibody overnight at 4℃, and then incubated with second antibody at 37℃ for 30min, followed by hematoxylin counterstaining of nuclei and coverslipping with re-sin. Following each step , coverslips were rinsed with 0.01 mol/L PBS three times, each for 5 min. These coverslips were observed after mounting and pictures were taken.

1.4 Detecting the relative quantity of *NANOG* mRNA by RT-PCR Total RNA of SHG-44, U87 MG and U251 of both control groups and experimental ones were extracted with Trizol reagents according to the manufacture's instruction. Briefly, about 1×10^7 cells were collected in 1.5 mL of denaturing sulotion. After two chloroform extraction, RNA was precipitated with isopropanol, washed with 75% ethanol and air-dried. The extracted total RNA was re-suspended in RNAsecure™ and stored at －20 ℃. The concentration of total RNA was mea sured with Biophotmeter (Eppendorf, Hamburg, Germany). The quality of total RNA was verified by agarose gel electrophoresis.

Single-stranded cDNA was synthesized from 5 μg total RNA using PrimeScript RT reagent(TaKaRa). PCR primers were: human *NANOG*, forward, 5′-TCA GGG CTG TCC TGA ATA AGC-3′, reverse, 5′-CCG ACT GTA AAG AAT CTT CAC CTA TG-3′(156 bp); GAPDH, forward, 5′-CCA CCC ATG GCA AAT TCC CAT GGC A-3′, reverse, 5′-TCT AGA CGG CAG GTCAGG TCC ACC-3′(597 bp). PCR condition were: the initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of amplification with 30 s of denaturation at 94 ℃, 30 s of annealing at 55 ℃ and 40 s of extension at 72 ℃, and a final extension at 72 ℃ for 10 min. PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide staining. Band intensity was quantified by using of Gene Tool Software. The gray values of bands were normalized relative to those of *GAPDH*. The gray values were expressed in relation to that of control and presented as $\bar{x}\pm s$ from 3 independent experiments.

1.5 Statistical analysis All experimental data were expressed by $\bar{x}\pm s$. The software of SPSS version 17.0 was used for data analysis. One-way ANOVA with was used to compare the positive rate of NANOG protein and the quantity of *NANOG* mRNA of different cell lines and groups. $P<0.05$ was considered statistical significance.

2 Results

2.1 Immunocytochemistry expression of NANOG in reduction after treated glioma cell lines ATRA Immunohistochemical results showed that NANOG protein was expressed in all glioma cell lines SHG-44, U87 MG, U251, shown brownish yellow or yellow solid particles which were located mainly in the nuclei(Fig. 1 in cover page 2). Randomly, 20 microscopic fields were selected on each coverslip and investigated under the microscope to calculate the percentages of NANOG-positive cells. The calculation formula was: percentage of NANOG-positive cells＝NANOG-positive cells/total cells×100%. The positive rates of NANOG protein in SHG-44, U87 MG, and U251 in control groups were (65.5±3.0)%, (64.8±8.0)%, (64.5±1.2)%, respectively. The analytical results showed that the differences was not statistically significant($F=0.19$, $P=0.829$). However, in the experimental groups, the positive

rates of NANOG expression were (36.5±7.3)%, (35.5±7.9)%, (35.2±6.1)%, respectively. Statistical analysis showed that the difference between the control group and the experimental one of each cell line was statistically significant ($F_{SHG-44}=259.1$, $F_{U87}=129.5$, $F_{U251}=431.8$, $P_{SHG-44}=0.0$, $P_{U87}=0.0$, $P_{U251}=0.0$) (Fig. 2). Immunocytochemical results were in agreement with the reports of many literature. In addition, our results showed NANOG protein also exsisted in cell plasma except being expressed in the nucleus. Further more, we also found that, there were more axons of these cells treated with ATRA compared with those untreated.

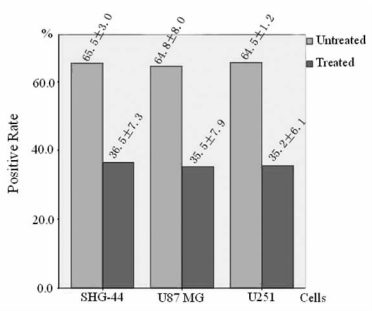
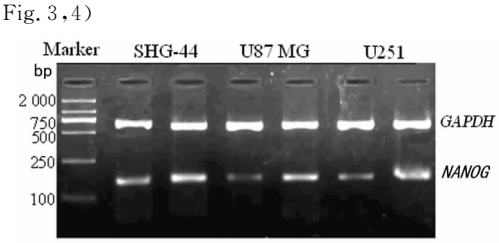


Fig. 2 The statistical analysis of positive rate of NANOG protein in SHG-44, U87 MG and U251cells.

2.2 RT-PCR detection *NANOG* mRNA were all expressed in SHG-44, U87 MG, U251, and all target gene bands were less bright than that of the internal reference *GAPDH*, and both *NANOG* gene and internal reference *GAPDH* bands appeared at correct position, at 156 bp and 596 bp. After analysis by Gene tool software, it was found that the relative expression levels of *NANOG* mRNA in control groups were 0.636 8±0.039 9, 0.642 1±0.063 7, 0.651 6±0.044 4, respectively, which statistical analysis showed the difference of *NANOG* mRNA in different cell lines was not statistically significant ($F=0.427$, $P=0.662$), which was consistent with the results of immunohistochemistry. However, in experimental groups, the relative level of *NANOG* mRNA in SHG-44, U87 MG, U251 were 0.458 3±0.079 1, 0.255 1±0.079 3, 0.333 1±0.054 0, which statistical analysis showed that the difference among the same cell line was significant ($F_{SHG-44}=77.8$, $F_{U87}=277.9$, $F_{U251}=398.1$, $P_{SHG-44}=0.0$, $P_{U87}=0.0$, $P_{U251}=0.0$) which were agreement with the results of immunocytochemistry. (Fig. 3, 4)



GAPDH was used as the internal reference gene which was 597 bp. *NANOG* was target gene which was 156 bp, and the brightness of all target genetic bands was lower than that of GAPDH.

Fig. 3 Detecting the level of *NANOG* in SHG-44, U87 MG and U251 cells.

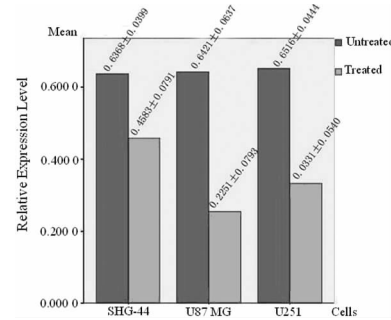


Fig. 4 The statistical analysis of *NANOG* mRNA.

3 Disscusion

Intracranial glioma is a common malignancy, accounting for 33.3%－58.9% of brainl tumors, 43.5% on average, with strong invasiveness, can easily invade the surrounding normal brain tissue, and the boundaries between them is not clear, often encroach several lobes and deep brain structures as well as the contralateral brain hemisphere. It is difficult to remove completely by surgery. Glioma, with high malignancy, fast growth and short course, has the only 3.5 days of the average cell cycle, which is a serious threat to human health for its recurrence soon. Although combined treatments, including surgery, radiation therapy, chemotherapy and immune therapy, have been applied, the curative efficacy is disatisfactoried. And the five-year survival rate of glioma postpoperative patients is still low. which is the problem of neurosurgery, and one of the important issues of medical research.

NANOG is specifically expressed in embryonic stem cell[8,14-16], which can not be detected first until at the period of morula stage, and there is no obvious expression at the early cleavage stage. To the early blastocyst, NANOG expression is limited to the inner cell mass, there is no expression in the trophectoderm. In the late blastocyst, *NANOG* mRNA is expressed in epiblast, and doesn't exsist in the primitive endoderm. Once embryonic stem cell transplants, NANOG is only expressed in epiblast, especially expressed most near the front[8,15-17], when it accesses to gastrula, the NANOG expression begins to quickly reduce[8,17]. NANOG is also expressed in Reconstruction blastocysts formed by nuclear transplantation and the cells formed by cell fusion with embryo stem cell, which is closely related to nuclear gene reprogramming[18-20]. In addition, the embryonic stem cell with heterozygous NANOG (+/−) gene are easily leading to differentiation compared to embryonic stem cells homozygous, indicating that differentiation of embryonic stem cells are dependent on the level of expression of NANOG [21-23].

Cancer stem cell theory[24] makes us realize that the formation of malignant tumor is derived from a series of genetic mutations of stem cells, gifted the ability to abnormal self-renewal and further proliferation and differentiation The doctrine of Cancer stem cells theory is that there is only a small bit of cell with the ability to infinite proliferation, self-remewal and multilineage differentiation potential which is the source of me-

tastasis and recurrence of tumor, and meanwhile is also a major target for Cancer therapy in the future[25], and while most of the tumor cell originated from cancer stem cells are easily killed by conventional radiotherapy and chemotherapy. The residue of stem cells may lead to recurrence and metastases of tumors via the blood to reach other parts. That is , current oncology treatment based mainly on chemotherapy and radiotherapy only find to kill the cell after the differentiation of cancer stem cells and make the tumor cell number decrease, so we can observe tumor shrinkage, whilethere is no significant effect on cancer stem cells[26-30], it is difficult for patients to get long-term survival.

With further research, it was found that, in addition to the expression of NANOG in embryonic stem cells, it was also expressed in some somatic tumors, such as breast cancer, glioma, prostate cancer, liver cancer, bladder cancer, stomach cancer, cervical cancer[8-10], and the higher grade of tumor, the more its expression, and the worse the prognosis, and high-grade tumors with the features of ESCs. These findings indicated that there existed some cells in the tumor maintaining the growth and self-renewal of tumor, which was constant with cancer stem cells theory. At the same time, it is also found the expression of NANOG in some tumor cell lines such as ovarian cancer cell line SK-O-V3 and breast cancer cell line MCF-7[18]. And NANOG together with CD44 and hyaluronic acid to form complexes, initiate the downstream ESC gene of NANOG such as Sox2, Rex1 etc, and form pathway with the Stat3 for the activation of the expression of its downstream some drug-resis-tant genes such as MDR1, co-promote the proliferation and multi-drug-resistance[31-32]. Fujii[33] detected the expression of NANOG in human sarcoma cell lines, including osteosarcoma MG63, Ewing′s sarcoma and fibrosarcoma HT1080 HTB166, which may be the reason of drug-resistance to cisplatin, adriamycin and other chemotherapy.

Ben-Porath et al.[34] reported that brain tumor stem cells (BTSCs) have some similarities with human ESCs on the biological characteristics, both had the ability of self-renewal and differentiation, and some of the regulatory function of signaling pathways in ESCs also played an important role in BTSCs. The transcriptional factor NANOG and its target gene, located in upstream of self-renewal and differentiation of ESCs balance regulatory network, were also expressed in glioma tissue, and was closely related to differentiation and the pathological level, while, in normal brain tissue, there is no expression of NANOG, suggesting that, in BTSCs, NANOG played the similar role in ESCs, which also may be located in upstream of re-gulatory network of self-renewal and differentiation balance. Therefore, NANOG, as a target, inducing the differentiation of BTSCs, the desired effect may be acquired. In addition, it was also found in our experiment that NANOG is significantly expressed in glioma cell lines SHG-44, U87 MG, U251, which was in agreement with the above reports. This finding suggests the existence of brain tumor stem cells with the characteristics of cancer stem cells, in glioma, maintaining the continuous proliferation and renewal of glioma cell lines.

Induction of differentiation therapy is a new field of cancer treatment, and has become a new hotspot of international can cer treatment. In recent years, the tumor molecular biological studies have shown that: cancer (including brain tumors) is a cell differentiation disorder, and the specific gene caused the inhibition of cell differentiation under certain conditions, is reversible. Induction of differentiation therapy is based on the above knowledge, it can relieve the developmental defect of tumor cells, and promote its further differentiation, and reverse its malignant phenotype of proliferation, invasion, metastasis, and so on. Eventually make the tumor cell become a normal or close to normal cells, in order to become a new approach of controling tumor. ATRA is one of main inducers applied to glioma. It was found that ATRA can induce the redcution of the expression of NANOG in glioma cell lines U87G, promoting the differentiation of the cell lines. In our experiment, it was also found that ATRA can induce the expression decreased of NANOG in U87 MG, U251, SHG-44 cell. In addition, we also found that cells treated with ATRA became more flat, and had more axons which became longer than those untreated. These are morphological signs of differentiation of all glioma cell lines after application of ATRA. Experimental results verify our hypothesis that ATRA can reduce the expression decreased of NANOG in three glioma cell lines, and lead to the differetiation of tumor cells.

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