

## RESEARCH ARTICLE

# All-trans-retinoic Acid Promotes Iodine Uptake Via Up-regulating the Sodium Iodide Symporter in Medullary Thyroid Cancer Stem Cells

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### Abstract

Recently, the main therapy of medullary thyroid cancer (MTC) is surgical, but by which way there is a poor prognosis with a mean survival of only 5 years. In some cases, some researchers found that it is the medullary thyroid cancer stem cells (MTCSCs) that cause metastasis and recurrence. This study aimed to eradicate MTCSCs through administration of all-trans-retinoic acid (ATRA). Here we demonstrate that MTCSCs possess stem-like properties in serum-free medium. The ABCG2, OCT4 and sodium iodide symporter (NIS) were changed by ATRA. Additionally, we found that ATRA can increase the expression of NIS *in vivo*. All the data suggested that ATRA could increase the iodine uptake of MTCSCs through NIS.

**Keywords:** ATRA - medullary thyroid carcinoma stem cells - the capability of radioiodine uptake - <sup>131</sup>I

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### Introduction

MTC accounts around 5%-10% of thyroid cancer, and the death rate is more than 10%. It has a poor capability of iodine uptake which limits the way of therapy. Furthermore, many people believe that MTCSCs may play significant roles in metastasis and recurrence of MTC.

Cancer stem cells (CSCs) have been reported in several solid tumors and in cancer cell lines (Reya et al., 2001; Reya et al., 2001; Ailles and Weissman, 2007; Lobo et al., 2007). Recently, people believed that only a few cells of CSCs have the capacity to sustain the growth of tumor. And this subpopulation of cells are capable of self-renew, proliferate and differentiate into other tumor cells (Jiang et al., 2012; Tabarestani and Ghafouri-Fard, 2012; Francipane et al., 2013). It also expresses high levels of ABCG2 and OCT4, which may cause their resistance to the conventional therapies of cancer (Tabarestani and Ghafouri-Fard, 2012; Zhang et al., 2012). Thus, if we find a way to eliminate the CSCs, we may cure the cancer.

All-trans-retinoic acid (ATRA) has the capacity of inducing differentiation, inhibiting proliferation and promoting apoptosis of many tumors and CSCs. It can induce differentiation of acute promyelocytic leukemia stem cells, and increase 5-year survival rate with arsenic trioxide (Cunha De Santis et al., 2007; Tomita et al., 2013).

The sodium iodide symporter (NIS), as the first step in thyroid hormone biosynthesis, mediated thyroidal I-transport from the bloodstream to the colloid (Schmutzler et al., 1997; Foujilas et al., 2009; Damle et al., 2011). This

supports production of thyroid hormones tri-iodothyronine (T3) and thyroxine (T4), and facilitates the use of radiolabelled iodide for imaging and therapy of thyroid disease (Doha'n et al., 2003). As reported, the expression of NIS was regulated by ATRA (Ryan et al., 2011).

In this study, we try to increase the capability of iodine uptake of MTCSCs through ATRA, which may provide new ideas for the clinical treatment of the MTC, even of thyroid cancer.

### Materials and Methods

#### Cells cultures

Human medullary thyroid cancer cells line TT were cultured in RPMI 1640 media with 10% fetal bovine serum (Gibico, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Beyotime, China). The cells were grown at 37 °C under 5% CO<sub>2</sub>, with medium change every 2-3 days.

#### Sphere formation

Tumor spheres were generated by placing TT cells into serum-free DMEM/F12 medium containing bFGF (20 ng/ml, Peprotech), EGF (20 ng/ml, Peprotech), insulin (5%, sigma-aldrich), and BSA (0.4%, Biosharp). Every 3-4 days all the factors were added. Ten days later, some cells would form spheres.

#### Colony formation analysis

To evaluate the proliferation of TT cells and sphere

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cells, a colony formation assay was performed. TT cells and sphere cells were planted to 6-well, with  $10^3$  of each well and medium being changed every 3 days. Two weeks later, washed it with ice-cold PBS and fixed it for 5 minutes with methanol, stained with Giemsa dye, and observed under light microscope (n=500 cells).

#### FCM analysis

The cells include TT and sphere cells were incubated with saturating amounts of OCT3/4-PE or ABCG2-PE, which came from Santa Cruz (USA), for 20 min at 4°C in the dark. Then, cells were washed and analyzed on FCM.

#### Nude mouse xenograft model

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Standard of China. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Chongqing Medical University (Reference Number: CQMU 2013005). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

For *in vivo* tumorigenesis assay,  $10^3$ ,  $10^4$ ,  $10^5$  and  $10^6$  cells of the TT and TT sphere cells were athymic nude mice (NOD/SCID, Beijing HFK Bioscience, LTD, China), respectively. In this study, every group had 3 nude mice. 2 weeks later, measured the diameter of tumors, which exceeds 5 mm.

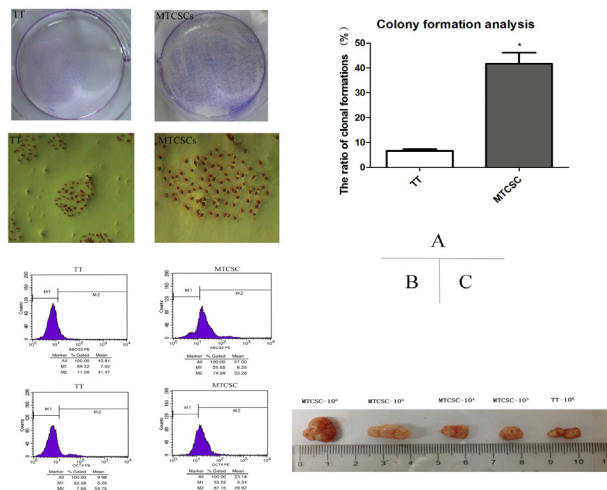
#### ATRA

Collected the TT cells and MTCSCs, which are in exponential phase, and plated them in 96-well,  $10^4$  per well. 24 hours later, adding different concentrations of ATRA ( $1 \times 10^{-7}$ ,  $2 \times 10^{-7}$ ,  $5 \times 10^{-7}$ ,  $1 \times 10^{-6}$ ,  $2 \times 10^{-6}$  M) (Sigma-Aldrich), the blank control group add with the same amount of absolute ethanol respectively. 24 hours later, all the cells were observed under a microscope.

All of the cells were used to measure the inhibition ratio by the methyl-thiazolyl-tetrazolium (MTT) test. Briefly, the medium containing 5 mg/ml MTT was added to each well and incubated at 37°C for 6 hours. The product was dissolved in Dimethyl sulfoxide (DMSO) for 10 min and the plates were read at 490 nm using a plate reader. The inhibition ratio was expressed as  $1 - \text{experimental/control}$  and calculated the half maximal inhibitory concentration of a substance (IC50) with the inhibition ratio.

#### Western blot analysis

Collecting the TT cells and MTCSCs with or without ATRA for 24 hours, the protein extracts were prepared to use a nuclear extraction kit. Twenty microliters of sample were boiled at 95°C for 5 minutes and centrifuged at 12000 r.p.m. for 5 minutes at 4°C. The sample was separated in a 10% SDS-polyacrylamide gel electrophoresis gel and transferred to Polyvinylidene Fluoride (PVDF) membranes by a wet transfer system. The membranes were washed by TBS containing 0.1% Tween (TBST) twice. And incubated with the primary antibodies were used: mouse anti-human ABCG2, mouse anti-human OCT4, mouse anti-human NIS and mouse anti-human GAPDH (Abcam, UK) for overnight at 4°C. The membranes were



**Figure 1. Characterization of TT Cells and MTCSCs.**

(A) colony formation analysis, the ratio of colony formations, it showed that the ratio of MTCSCs was  $41.69\% \pm 4.45\%$  with  $6.65\% \pm 0.69\%$  of TT. The results are presented as  $\bar{x}(-) \pm s$  of eight independent experiments, n=8. \* $p < 0.05$ . (B) the maker of stem-like were detected by FCM, (C) The nude mouse xenograft model

incubated with Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG (ZSGB-BIO, China) for 1 hour after washing with TBST 3 times. At last, ECL detection system (Thermo) was used to detect the four proteins.

#### <sup>131</sup>I uptake assay

To determine whether the ATRA can influence radioactive iodide uptake, iodide uptake assay was performed as described by Weiss et al. (1984) with some modifications. The TT cells and MTCSCs, which were cultured 24 hours with ATRA, packed it in centrifuge tubes which have 1 ml of Hanks' balanced salt solution (HBSS) with  $10^5$  cells. Added 37 kBq of <sup>131</sup>I to each tube and washed with ice-cold HBSS 2 hours later in 37°C. Their radioactivity of the cells was measured with a gamma counter. The counts were normalized the number of viable cells.

#### Statistical analysis

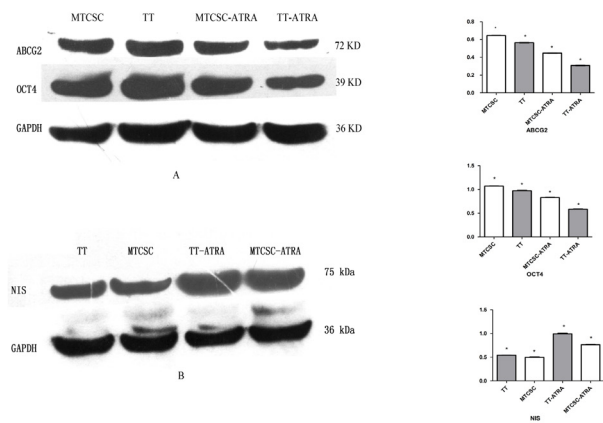
Statistical analysis was performed with Graphpad Prism 5 software. Numerical data were expressed as means  $\pm$  SD. Significance was concluded at  $p < 0.05$ .

## Results

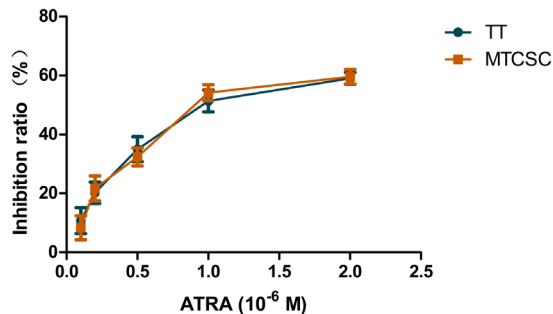
#### Characterization of TT cells and TT sphere cells

To determine the cells which were separated by serum-free culturing had some stem-like feature, the colony formation analysis, xenograft tumor model and FCM were performed. As revealed by colony formations assay, we found that the PE of TT sphere cells was  $41.69\% \pm 4.45\%$  and  $6.65\% \pm 0.69\%$  of TT (Figure 1A). It means that the TT sphere cells had more proliferation than TT cells. Addition, when detected the maker by FCM, the expression of ABCG2 and OCT4 were 74.98% and 67.15% in TT sphere cells respectively, while them were 11.06% and 7.66% in TT cells (Figure 1B).

Moreover, in the tumorigenesis assay, we found that all the group of TT sphere cells generated xenograft



**Figure 2. Expression of OCT4, ABCG2 and NIS.** Western blot was used to assay the change of OCT4, ABCG2 and NIS with treated or not. TT and MTCSCs represented the cells without ATRA, TT-ATRA and MTCSC-ATRA were exposed after 24h to ATRA. All the results are presented as  $\bar{x}(\pm)s$  of five independent experiments,  $n=3$ . \* $p<0.05$



**Figure 3. The Inhibition Ratio of TT and MTCSCs with Treated by ATRA for 24 h.** It is shown that inhibition ratio was positively correlated with the concentration. And all the values were readied at Table 1

tumor model, and the tumorigenesis only  $10^6$  cells of TT (Figure 1C). The xenograft tumor model was considered successful formed while the tumor was more than 5mm in diameter.

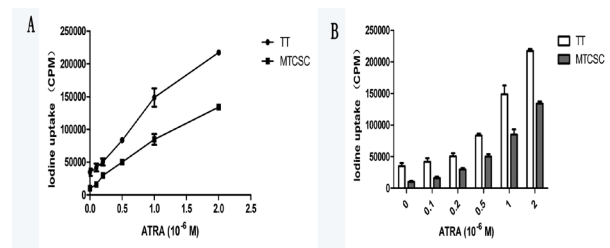
#### Expression of OCT4, ABCG2 and NIS

In order to prove the proteins of TT cells and TT sphere cells are different, the western blot was used. We assayed the expression of OCT4 and ABCG2 of TT cells and TT sphere cells (Figure 2A), In TT sphere cells, both of them had a higher expression levels than it in TT cells (Figure 2A). In summary, the TT sphere cells, which had the ability to self-renew, proliferate, formed xenograft tumor and expressed the stem-like maker, were considered as medullary thyroid cancer stem cells (MTCSCs). Interestingly, when we detected the NIS, the expression levels of TT sphere cells were lower than TT cells (Figure 2B).

#### Treatment of ATRA

The plate reader showed that inhibition ratio was positively correlated with the concentration. It was proved that the capacity of inhibition would be enhanced as the concentration increased (Figure 3). And the  $IC_{50}$  of MTCSCs was  $1.06 \times 10^{-6}$  M,  $1.10 \times 10^{-6}$  M of TT cells (Table 1).

Addition, the western blot showed that OCT4 and



**Figure 4. Iodine Uptake.** The TT and MTCSCs cells were administrated by ATRA, and CPM were detected by the gamma counter. All the results are presented as  $\bar{x}(\pm)s$  of fifteen independent experiments,  $n=15$ . \* $p<0.05$

**Table 1. Inhibition Ratio of TT and MTCSC with Treated by ATRA ( $\bar{x}(\pm)s$ )**

The concentration of ATRA( $10^{-6}$ M)	n	TT(%)	MTCSC(%)
0.1	8	10.79 $\pm$ 4.40*	8.37 $\pm$ 4.07*
0.2	8	20.20 $\pm$ 3.64*	21.67 $\pm$ 4.24*
0.5	8	34.98 $\pm$ 4.23*	32.34 $\pm$ 3.07*
1	8	51.33 $\pm$ 3.66*	54.17 $\pm$ 2.67*
2	8	59.02 $\pm$ 1.98*	59.59 $\pm$ 2.43*

$IC_{50}$  values were determined by these data. The  $IC_{50}$  for TT is  $1.10 \times 10^{-6}$  M, and MTCSC is  $1.06 \times 10^{-6}$  M. \* $p<0.05$

ABCG2 of MTCSCs were down-regulated the same as TT cells, after treated by ATRA with  $1 \times 10^{-6}$  M (Figure 2A). But, NIS was up-regulated as the same treated (Figure 2B).

#### Capability of iodine uptake

The capability of iodine uptake of MTCSCs and TT cells had changed after being treated by ATRA with difference concentration. The capability of MTCSCs and TT cells was increased as the concentration of ATRA increased. And at the concentration of  $IC_{50}$ , the capability of MTCSCs increased 8.30 times and 4.27 of TT cells (Figure 4).

## Discussion

Cancer stem cells (CSCs) are thought to represent a small sub-population of cells presented in most tumors, possess the ability to self-renew, proliferate and produce progenitor cells that can reconstitute and sustain tumor growth (Collins, 2002; Christina Zechel, 2005; Rashidi et al., 2013). Moreover, CSCs also play a key role in cancer metastasis, cancer recurrence, and cancer drug resistance (Hadnagy, 2006; Jordan et al., 2006; Lin, 2011).

In this study, we have demonstrated that MTCSCs could be isolated by serum-deprivation, to acquire some characterized of stem-like cells. In this population of cells, we found that the MTCSC had high expression levels of ABCG2 and OCT4, observed by FCM. And the ABCG2, which was believed as a gene of drug resistance, of MTCSCs have a high level. These may explain why CSCs are resistant to chemotherapy and radiotherapy.

The colony formations test showed that the capacity of MTCSCs, namely self-renewal and proliferation, were more powerful than that of TT cells. And the level of stemness protein (OCT4), which is considered key genes for the production of murine and human induced pluripotent stem cell (Bao et al., 2006; Martin et al., 2008), was higher



in MTCSCs than TT cells. Moreover, the tumorigenesis of MTCSCs can cause the tumor when planted in nude mice only  $10^3$ , as previous reported (Baumann et al., 2008). We found difference concentration of ATRA could cause the different effects in TT cells and MTCSCs. At present, the regulation and function of NIS have been well know in thyroid tissue. This may make a better understanding of thyroid diseases and therapy using iodine (Doha'n and Carrasco, 2003). The NIS, as the first step in thyroid hormone biosynthesis, mediated thyroidal I- transport from the bloodstream to the colloid (Segalla et al., 2003; Takahashi and Yamanaka, 2006; Yu et al., 2007; Tseng et al., 2012). It can provide the basis for the effective therapeutic management of thyroid cancer with radioiodine. In this study, we find that the NIS was up-regulated by ATRA. Its ability to mediate iodine uptake is demonstrated by the test of iodine uptake. With the ATRA administrated, the capability of iodine uptake was increased.

Overall, in this study, we certificated that ATRA can affect the capacity of iodine uptake through up-regulating the expression of NIS (Biermann et al., 2011; Elola et al., 2011). Next, we will demonstrate that ATRA can promote the eradication of MTCSC by iodine in vivo. In conclusion, in this study, we used the ATRA to treat the MTCSCs and TT cells, revealing that the ATRA can induce the differentiation of MTCSCs and TT cells, and also increase the capacity of iodine uptake, which will be helpful to treat MTC for clinic.

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