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OVERCOMING THE LACK OF EXPRESSION OF THE SODIUM-IODIDE SYMPORTER PROTEIN IN ANAPLASTIC THYROID CANCER STEM CELLS BY TARGETING THE PHOSPHATIDYL INOSITOL 3-KINASE SIGNALING PATHWAY

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ABSTRACT

Background: Anaplastic thyroid carcinoma (ATC) is rare, but it is the most aggressive thyroid cancer. Cancer stem cells (CSCs), which have self-renewal ability and can generate multi-lineage cells, are potential causative agents for ATC recurrence. To assess the role of CSCs in ATC, we evaluated the sodium-iodide symporter (NIS or SLC5A5) and Phosphatidyl Inositol 3-Kinase (PI3K) mRNA levels in the (CD133pos) CSC fraction of ATC cell lines.
Methods: Using magnetic cell sorting, we isolated (CD133pos) CSCs from three ATC cell lines. After verification of the cell purity by flow cytometry, the NIS and PI3K mRNA levels were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). We performed immunocytochemistry to determine NIS protein localization after treated these cells with LY294002, a PI3K inhibitor.
Results: The qRT-PCR results revealed that the (CD133pos) CSCs extracted from the ATC cell lines had lower NIS mRNA expression and higher PI3K mRNA expression than normal human thyrocytes. In addition, the cells were capable of expressing the NIS protein, but only after PI3K inhibitor treatment.
Conclusion: High PI3K levels in (CD133pos) CSCs may impair NIS gene and protein expression. The lack of NIS gene and protein expression in these cells may be a critical reason for radioiodine therapy resistance in patients with ATC. Targeting the CSCs along with conventional thyroid cancer therapy may be a useful molecular curative approach for treating aggressive carcinomas, especially ATC, in the future.

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INTRODUCTION

Although anaplastic thyroid carcinoma (ATC) is rare, it is the deadliest thyroid cancer. It is characterized by swift growth, recurrence, and resistance to conventional thyroid cancer therapy such as radioiodine therapy. Typically, after surgical removal of the thyroid tumor, radioactive iodine therapy is used for thyroid cancer management (Cabanillas *et al*.2016). Several distinct factors are involved in the efficacy of this

therapy id est sodium-iodide symporter (NIS) protein. This glycoprotein is fundamental for iodide accumulation in thyrocytes. It is localized on the basolateral membrane of thyrocytes and pumps two sodium molecules along with one iodide molecule from the bloodstream into these cells to facilitate thyroid hormone production (Paroder-Belenitsky *et al*. 2011; Ozcan Kara *et al*. 2014). Earlier studies have indicated that several factors can be involved in NIS protein expression and function, such as Phosphatidyl Inositol 3-Kinase (*PI3K*) gene mutation (Bozorg-Ghalati and Hedayati 2015).Kogai *et al*. reported that PI3K inhibition affects *NIS* gene expression in rat thyrocytes and human papillary thyroid cancer cells (Kogai *et al*. 2008). In addition, cancer stem cells (CSCs) are potential agents that drive cancer recurrence (Lin 2011; Todaro *et al*. 2010; Li *et al*. 2016). They are characterized by several surface markers, including CD133. CD133 acts reciprocally with distinct signaling pathways, like PI3K/Akt, Wnt/β-catenin, Notch, and NF-κB, to promote stem cell marker expression, block cancer cell differentiation, and inhibit apoptosis. Therefore, cancer cells with CD133 expression may have self-renewal ability, tumorigenic potential, and multi-drug resistance (Wei *et al*. 2013; Zobalova *et al*. 2008; Xi *et al*. 2016). Ke *et al*. discovered that the $CD133^{pos}$ CSCs fraction of ATC cell lines contain undifferentiated cells and radioresistant. These cells are likely to play a critical role in cancer cell survival after radioiodine therapy (Ke *et al*. 2013). Molecular targeting and differentiation therapies are hot topics for treating aggressive and recurrent malignant cancers like ATC (Bozorg-Ghalati and Hedayati 2017). Increasing information about the cell cycle, gene mutations, and the molecular signaling of CSCs is beneficial for the development of this therapeutic approach (Decaussin-Petrucci *et al*. 2015). Given the scant data regarding thyroid CSCs, particularly an unpublished paper on their *PI3K* gene expression level, we conducted this study with emphasis on the *PI3K* signaling transduction pathway in $CD133^{pos}$ cells in ATC cell lines. We also evaluated the expression levels of the *PI3K* and *NIS* genes in these cells, and investigated the effects of PI3K inhibition on NIS protein expression and localization.

MATERIALS AND METHODS

Ethics Statements: The research protocol was endorsed (approval no. 6066) by the Ethics Clearance Committee of Shahid Beheshti University of Medical Sciences and managed in accordance with international policies established by the Declaration of Helsinki.

ATC cell lines and cell culture: The ATC cell line 8305C was purchased from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran, Tehran, Iran). Two other ATC cell lines (SW1736 and C643) were generously provided by Dr. Vahid Haghpanah (Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences, Tehran, Iran). We cultivated and maintained them at 37°C with 5% CO2 in RPMI 1640 GlutaMAX™ medium (Biowest, Nuaillé, France) with 10% fetal bovine serum (Gibco™, EU-Approved, South American), 1% penicillin-streptomycin (Biowest) and 1% non-essential amino acids (Biowest).

Magnetic-activated cell sorting assay: A magnetic-activated cell sorting (MACS)® Human CD133 Micro Bead Kit – Tumor Tissue, (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to separate $CD133^{pos}$ CSCs from the ATC cell lines. The cell lines were cultured, then harvested using trypsin–EDTA (Sigma-Aldrich, Missouri, USA), and centrifuged at 300 ×g for 10 min. The cell pellets were resuspended in 60 µl of MACS buffer (Miltenyi Biotec), 20 µl of FcR Blocking Reagent (Miltenyi Biotec), and 20 µl of CD133 Micro Beads, then incubated for 15 min at 4 °C under slow rotation. Then, the cells were centrifuged (300 ×g, 10 min) and resuspended in MACS buffer (500 µl). We applied the cell suspension to an LS column and collected the flow-through. Finally, we removed the column from the separator, placed it in a collection tube, and immediately flushed the magnetically labeled cells by firmly pushing the plunger into the column.

Flow cytometry

The flow cytometry assay was executed per the Miltenyi Biotec protocol. After centrifuging the cell suspensions (300 ×g, 10 min), we aspirated the supernatants and resuspended the cells in 100 µl of buffer plus 10 µl of the CD133 antibody (Miltenyi Biotec), then incubated the cells at 4 °C for 10 min. Subsequently, they were centrifuged (300 ×g, 10 min), resuspended in 100 µl of buffer, and analyzed on a flow cytometry instrument (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Treatment: The $CD133^{pos}$ CSCs were cultivated and treated with 20 µM LY294002 (Chemietek, Indianapolis, USA), plus 5 µg/ml bovine Thyroid-Stimulating Hormone (Sigma-Aldrich), for 24 or 48 h.

RNA isolation and cDNA synthesis: After treatment, the cells were harvested and their total RNA was extracted according to the YTA Total RNA Extraction Mini Kit protocol (Yekta Tajhiz Azma, Tehran, Iran). The purity/quantity, and integrity of the total RNA were determined by ultraviolet spectrophotometry and agarose gel electrophoresis, respectively. The Revert Aid First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific, Waltham, MA, USA) was used for cDNA synthesis.

Quantitative real-time polymerase chain reaction assay: The quantitative real-time polymerase chain reaction (qRT-PCR) assay was performed with the StepOne™ PCR thermal cycler system, version 2.3 (Applied Biosystems, Lincoln, USA). The PCR mixture contained 100 ng of cDNA, the RealQ PCR 2x Master Mix SYBR Green high ROX® (Amplicon, Stenhuggervej, Denmark), plus *PI3K* and *NIS* (*SLC5A5*) gene primers (Macrogen, Seoul, South Korea) (Bozorg-Ghalati *et al*. 2017a). The samples were loaded in 48-well optical plates in triplicate. To create a standard curve for each gene, a set of ten-fold serial dilutions of the internal standard was included in all runs. The control mixture consisted of the PCR mixture without cDNA. For each sample, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primer was used for *PI3K* and *NIS* genes normalization. All data were linear within this concentration range, with correlation coefficients of $r^2>0.999$.

Immunocytochemistry: Immunocytochemistry (ICC) was performed according to the Abcam protocol, with a few modifications. After treatment, the $CD133^{pos}$ CSCs were fixed in −20 °C pre-chilled 100% methanol (Merck, Darmstadt, Germany). After three washes with PBS (Sigma-Aldrich), the cells were incubated for 10 min at RT in PBS with 0.1% Triton X-100 (Sigma-Aldrich).

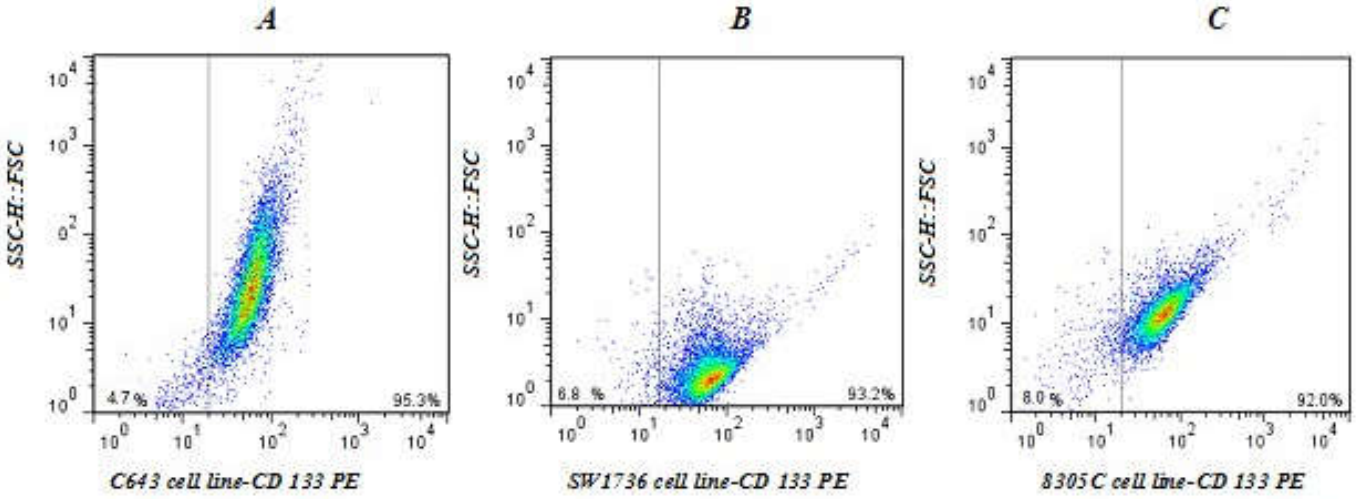

Figure 1. Flow cytometry results. The flow cytometry results verified the purity of the CD133^{pos} CSCs after isolation (A-C)

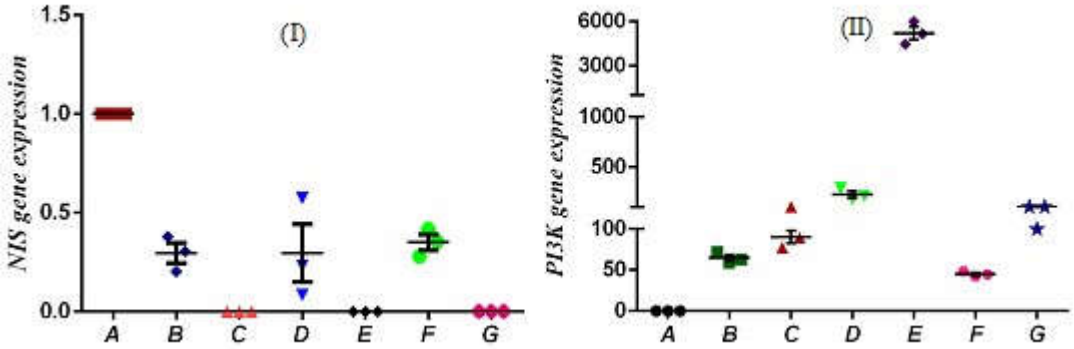

Figure 2. Evaluation of *NIS* (I) and *PI3K* (II) gene expression in the ATC cell lines and CD133^{pos} CSCs compared with in normal human thyrocytes by qRT-PCR. A: Normal human thyrocytes, B: C643 cell line, C: CD133^{pos} CSCs extracted from C643 cells, D: SW1736 cell line, E: CD133^{pos} CSCs extracted from SW1736 cells, F: 8305C cell line, G: CD133^{pos} CSCs extracted from 8305C cells. Expression of mRNA was calculated as a ratio to *GAPDH*.*P < 0.0001 by one-way ANOVA

Then, for 30 min at RT, they were incubated in 10% goat serum (Biowest) diluted in PBS with 5% BSA (Sigma-Aldrich) and 0.5% Tween® 20 (Merck). Following this step, the solution was discarded and the cells were incubated overnight at 4 °C in the NIS antibody (1:100 in PBS with 1% BSA and 0.1% Tween® 20). The cells were washed with PBS and incubated with goat anti-mouse IgG (diluted 1:200 in PBS with 1% BSA) for 1 h at RT in the dark. The washing step was repeated and the cells were incubated with the 3, 3'-diaminobenzidine (DAB) (Sigma-Aldrich) for 5 min. Finally, they were counterstained with hematoxylin (Sigma-Aldrich) and evaluated for NIS protein localization on an inverted microscope (Olympus, Tokyo, Japan).

Statistical analysis: The *PI3K* and *NIS* gene expression level data of qRT-PCR assay were analyzed with the Pfaffl formulas (Pfaffl 2001).The one-way analysis of variance (ANOVA) test and GraphPad Prism version 6.01 software (Graph Pad, San Diego, California) were used for the statistical analysis, including assessments of the differences in the *PI3K* and *NIS* mRNA expression levels. The differences were considered significant at $P < 0.0001$. The data are expressed as mean ± standard error of the mean (SEM) of three independent experiments executed in triplicate.

RESULTS

Verification of the separation purity: In order to verify the cell purities after separation, we performed flow cytometry (Figure 1).

The results of this test revealed that the CD133^{pos} CSCs were separated with 95.3% (Figure 1, A), 93.2 % (Figure 1, B), and 92.0% (Figure 1, C) purity from the C643, SW1736, and 8305C cell lines, respectively.

Evaluation of NIS and PI3K gene expression in the ATC cell lines, CD133^{pos} CSCs, and normal human thyrocytes: We assessed the levels of *NIS* and *PI3K* mRNA in the ATC cell lines, the CD133^{pos} cells, and normal human thyrocytes (Figure 2). We found that expression of the gene for *NIS* is lower in the ATC cell lines and the CD133^{pos} CSCs than in normal human thyrocytes (Figure 2, I). The relative expression levels (mean ± SEM) were 0.29 ± 0.04 (Figure 2, I, B), 0.29 ± 0.11 (Figure 2, I, D), and 0.35 ± 0.032 (Figure 2, I, F) in the C643, SW1736 and 8305C cell lines, respectively. The expression levels in the CD133^{pos} CSCs extracted from the C643, SW1736, and 8305C cell lines were 0.00019 ± 0.0003 (Figure 2, I, C), 0.00028 ± 0.0002 (Figure 2, I, E), and 0.00012 ± 0.0004 (Figure 2, I, G), respectively. The ATC cell lines and their CD133^{pos} CSCs have high expression of *PI3K* mRNA (Figure 2, II). The relative expression (mean ± SEM) was 64.76 ± 3.13 (Figure 2, II, B), 228.15 ± 26.77 (Figure 2, II, D), and 45.22 ± 1.42 (Figure 2, II, F) in C643, SW1736, and 8305C cells. The relative expression in the CD133^{pos} CSCs that were extracted from the C643, SW1736, and 8305C cell lines was 90.22 ± 6.36 (Figure 2, II, C), 5215.49 ± 359.86 (Figure 2, II, E), and 102.57 ± 1.06 (Figure 2, II, G), respectively.

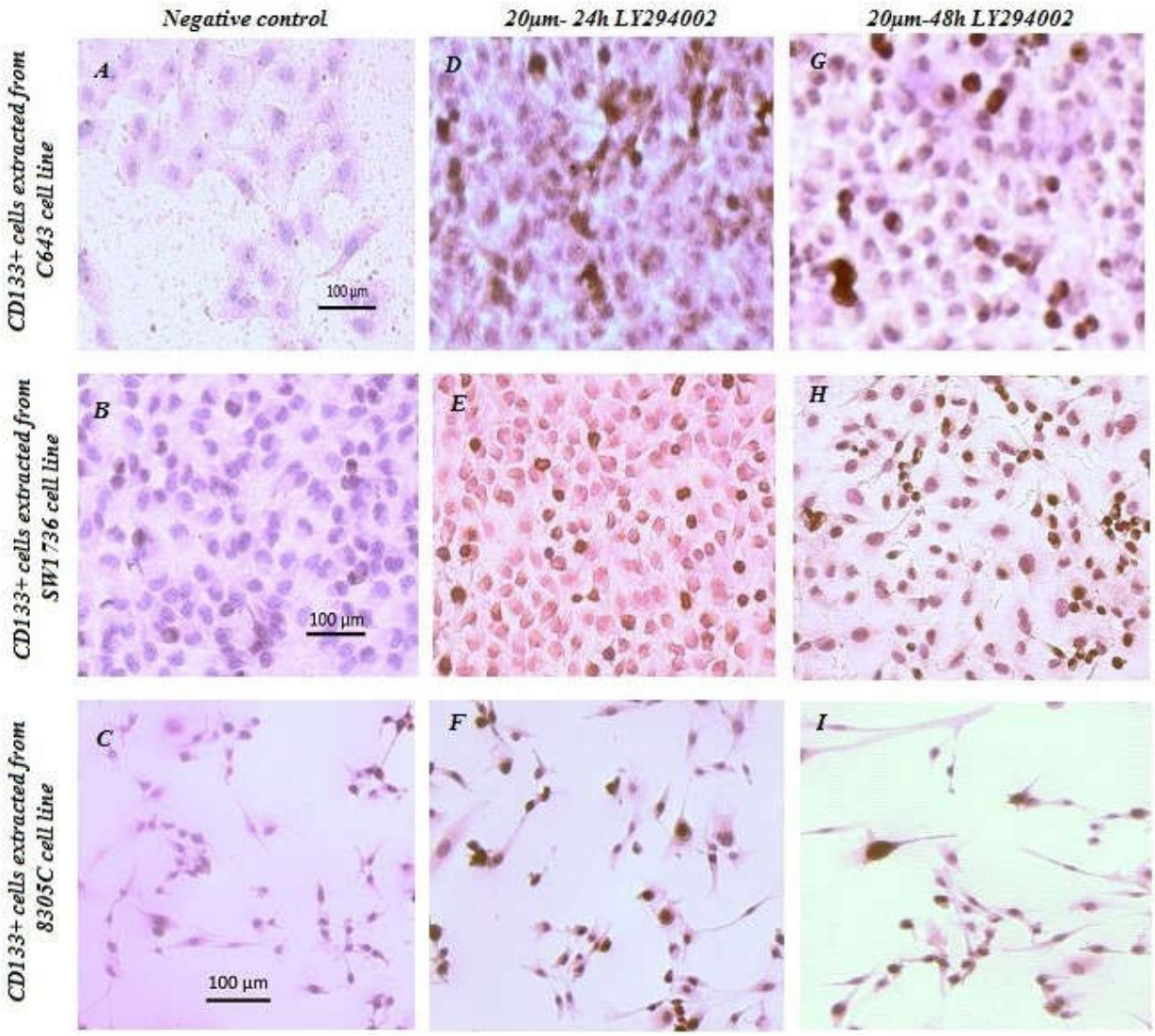

Figure 3. ICC for NIS in CD133^{pos} CSCs. We found that after 24 h (D-F) and 48 h (G-I) of treatment with LY294002, NIS protein expression increased in cytoplasm and plasma membrane of some these cells. We used cells without treatment as negative controls (A-C). Magnification, 200×

Evaluation of NIS localization in CD133^{pos} CSCs after PI3K inhibition: We performed ICC to evaluate NIS protein localization in the CD133^{pos} CSCs. As shown in Figure 3, LY294002 treatment increased the expression of the NIS protein in cytoplasm and plasma membrane of some these cells compared to before treatment.

DISCUSSION

CD133^{pos} cells are known as CSCs. They are rare among tumor cells, but, with their stem-like properties, they can induce cancer relapse (Ong et al. 2010; Huang et al. 2012; Bi et al. 2016). Various factors like gene mutations lead to a lack of expression of differentiation markers on these cells, which plays an important role in a poor prognosis (Bozorg-Ghalati and Hedayati 2016). The principal aim of this study was to evaluate the expression of *NIS* and *PI3K* mRNA in CSCs with CD133 surface expression from three ATC cell lines (C643, SW1736, and 8305C). Our first flow cytometry results (data not shown) indicated that the frequency of these cells in the cell lines is low (4% to 10%).

Due to these results, we performed multiple, sequential MACS separations using new LS columns to isolate highly pure CD133^{pos} CSCs. Our later flow cytometry results indicated that we could separate these cells to greater than 90% purity from the ATC cell lines. These results were obtained at the time of isolation, and the expression of CD133 on these cells was not tracked through the end of the current project. Given the controversial expression of this marker, it is likely that the degree of CD133 expression changed after isolation of the CSCs from the ATC cell lines (Haghpanah et al. 2016). However, following up on the expression of this marker in the isolated cells was not our purpose. Our objective was to investigate the expression of the *NIS* and *PI3K* genes in the CD133^{pos} CSCs separated from the ATC cell lines, and to evaluate the ability of the CSCs to express *NIS* protein before and after *PI3K* inhibitor treatment. The qRT-PCR results revealed that, compared to normal thyrocytes, the ATC cell lines have high and low levels of the *PI3K* and *NIS* genes, respectively. Surprisingly, the CD133^{pos} CSCs extracted from the ATC cell lines had even higher levels of *PI3K* (90 to 5215 folds) and lower levels of *NIS* mRNA compared to both the original ATC cell lines and normal thyrocytes. It seems that the CD133^{pos} CSCs appear to be reservoirs of *PI3K* gene

expression. Several previous studies demonstrated that *PI3K* gene mutation has a notable role in promoting ATC (García-Rostán et al. 2005; Charles et al. 2014). Therefore, our results suggest that $CD133^{pos}$ CSCs may have a crucial role in poor outcome for patients with ATC. In the current study, we focused on the PI3K signaling pathway and targeted *PI3K* in $CD133^{pos}$ CSCs. In our previous study, we reported that the $CD133^{pos}$ CSCs were able to express the *NIS* gene to 4.5-6 folds after treatment with 20μM LY294002 (Bozorg-Ghalati et al. 2017a). Thus, we treated the $CD133^{pos}$ CSCs with this PI3K inhibitor concentration and evaluated their NIS protein expression and localization by ICC assay. The ICC results indicated that NIS protein could be expressed in $CD133^{pos}$ CSCs after treatment. Thus, these cells were capable of expressing thyrocyte differentiation markers, like NIS. These results are in accordance with previous reports that showed that PI3K inhibition stimulated *NIS* gene and protein expression in thyroid cancer cells (Vlahos et al. 1994; Garcia and Santisteban 2002; Ke et al. 2014).

LY294002 acts as an inhibitor of the PI3K signaling cascade, and not as a transcript inhibitor (Liu et al. 2012); we believe it may have participated in a negative feedback loop on *PI3K* transcription, which decreased *PI3K* gene expression. Various studies have reported that NIS is overexpressed in the cytoplasm of many thyroid tumor cells (Gérard et al. 2003; Tonacchera et al. 2002). Gene mutations and other factors could impair the plasma membrane localization of NIS, leading to overexpression in the cytoplasm of thyroid tumor cells. However, our ICC results showed that before treatment, $CD133^{pos}$ cells did not express the NIS protein, neither in their cytoplasm nor in their plasma membranes. These results suggest that factors other than the surrounding thyroid tumor cells may be involved in NIS protein expression in CSCs. The evaluation of these factors and investigation of the reasons for this phenomenon are avenues for further research. In the present study, the functional efficiency of the NIS protein was not determined. Presumably, its post-translational modifications, membrane localization, and radiolabeled iodine uptake ability depend on several factors and hormones, like TSH (Kogai et al. 2008). Taken together, our data showed that the lack of *NIS* gene and protein expression in the $CD133^{pos}$ CSCs of ATC is a probable reason for radioiodine therapy resistance. In addition, with molecular targeting, such as PI3K inhibition, the thyrocyte differentiation marker NIS can be induced in these cells. Hence, understanding the cell cycle and signaling pathways in CSCs may be crucial to curing aggressive carcinoma (Yang et al. 2016; Bozorg-Ghalati et al. 2017b; Bozorg-Ghalati et al. 2017c). Our data suggest that targeting the CSCs along with the traditional thyroid cancer therapy may be a potentially useful approach for treating aggressive carcinomas, especially ATC, in the future.

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Conflicts of interest: The Authors declared no conflict of interest.

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