RESEARCH ARTICLE

Effects of a Phosphoinositide-3-Kinase Inhibitor on Anaplastic Thyroid Cancer Stem Cells

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Abstract

Background: Thyroidectomy, radioactive iodine therapy, chemotherapy, or their combination are treatments of choice for thyroid cancers. However, cancer stem cells (CSCs) may become resistant to therapy, and mutations in somatic genes affect radioiodine uptake. This study determined the effect of a phosphoinositide-3-kinase (PI3K) inhibitor on anaplastic thyroid CSCs. **Materials and Methods:** The magnetic-activated cell sorting assay was used for segregating CD133-positive CSCs from three anaplastic thyroid carcinoma (ATC) cell lines (C643, SW1736, and 8305C). After confirming the cells' purity by flow cytometry, they were treated with 5, 10, 20, or 25 μM LY294002, a PI3K inhibitor, and then evaluated at 24 and 48 h. The sodium-iodide symporter *(NIS)* mRNA level was determined using the quantitative real-time polymerase chain reaction. NIS protein expression was evaluated using western blotting. **Results:** The PI3K inhibitor, at different concentrations and times, increased the *NIS* mRNA level (1.30-6.17-fold, $P < 0.0001$). If the *NIS* mRNA level in LY294002-treated CD133-positive CSCs was increased more than 2-fold, the NIS protein content was detectable. **Conclusions:** CD133-positive CSCs isolated from ATC cell lines expressed NIS mRNA and protein after PI3K inhibition. Our findings suggest that molecularly targeted CSC therapy may improve the treatment efficacy of aggressive cancers like ATC.

Keywords: Anaplastic thyroid carcinoma- CD133 antigen- Phosphoinositide-3-kinase- Sodium-iodide symporter

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Introduction

At the present time, thyroidectomy followed by radioactive iodine (RAI) therapy, chemotherapy, or both are the treatments of choice in thyroid cancers (Fahrner et al., 2014; Fortuny et al., 2015). Most types of thyroid cancers, such as papillary and follicular thyroid cancers, can be treated successfully (Schmidbauer et al., 2017). However, advanced cancer types, such as anaplastic thyroid carcinoma (ATC), usually have no successful treatments. ATC is known as an aggressive, lethal, and recurrent malignancy that is resistant to routine treatments, especially RAI therapy (Pezzi et al., 2016).

Several factors are involved in effective RAI therapy including the sodium-iodide symporter (*NIS*) protein. This glycoprotein is formed in 13 putative transmembrane domains and is localized on the basolateral membrane of thyrocytes. It expedites iodine uptake in thyroid follicular cells and has a critical role in RAI therapy (Darrouzet et al., 2016; Ferrandino et al., 2016). The function of this protein can be affected by different factors, and by mutation in some somatic genes such as phosphoinositide-3-kinase (PI3K).

PI3K engages in several cellular activities including cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking (Bozorg-Ghalati et al., 2015). Several studies reported that PI3K silencing can induce expression of *NIS* (Kogai et al., 2008). In addition to gene mutations (Bozorg-Ghalati et al., 2016), cancer stem cells (CSCs) in thyroid tumors are associated with tumor metastasis, recurrence, and drug resistance (Nagayama et al., 2016; Bozorg-Ghalati et al., 2017a,b). Due to the unknown effects of a mutant *PI3K* on *NIS* in thyroid CSCs, the present study examined CSCs expressing the CD133 surface marker in ATC cell lines, and surveyed *NIS* gene/protein expression after PI3K inhibition.

Materials and Methods

Culture of ATC cell lines

Three ATC cell lines (SW1736, C643, and 8305C) were used in this study. The SW1736 and C643 cell lines were graciously supplied by Dr. Vahid Haghpanah

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(Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences, Tehran, Iran). The third cell line (8305C) was purchased from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). All cells were cultured at 37°C under 5% CO_2, in RPMI 1640 GlutaMAX™ medium (Biowest, Nuaillé, France) and supplemented with 10% fetal bovine serum (Gibco™, EU-Approved, South American), 1% penicillin-streptomycin, and 1% non-essential amino acids (Biowest).

Magnetic-activated cell sorting (MACS)

CD133-positive CSCs were isolated from the three ATC cell lines by using the MACS method. A MACS® human CD133 Microbead Kit-Tumor Tissue (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturers' protocol. After cultivation of the cell lines, they were harvested by trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, 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 microbeads, and incubated at 4°C for 15 min under a low rotator speed. Then, the cells were washed with MACS buffer, centrifuged at 300 × g for 10 min, and resuspended in MACS buffer (500 µL). LS columns (Miltenyi Biotec) that were fixed on the MACS separator magnet, were rinsed with MACS buffer (3 mL) and the cell suspensions were infused. After gathering the effluent from each LS column, they were removed from the MACS separator and placed into a new collection tube. Finally, by applying the MACS buffer (5 mL) and piston, the magnetically-marked CD133-positive CSCs were obtained.

Flow cytometry

A suspension (100 µL) containing 10^6 cells/mL was prepared. Then, 10 µL of the CD133 antibody (Miltenyi Biotec) were added, mixed well, and incubated at 4°C for 10 min. Subsequently, the cells were washed with MACS buffer and centrifuged at 300 × g for 10 min. Finally, the supernatant was aspirated and a suitable amount of buffer added for analysis of the cells by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Treatments

CD133-positive CSCs isolated from the three ATC cell lines (C643, SW1736, and 8305C) were treated with 5, 10, 20, or 25 µM LY294002 (a PI3K inhibitor) (Chemietek, Indianapolis, IN, USA) and 5 µg/mL bovine thyroid-stimulating hormone (Sigma-Aldrich) for 24 and 48 h. The treatment of 24 samples was repeated two times. Cells cultured without the inhibitor were used for the control group.

RNA isolation and cDNA synthesis

Total RNA was extracted from the treated cells according to the YTA Total RNA Extraction Mini Kit protocol (Yekta Tajhiz Azma, Tehran, Iran). The purity, quantity, and integrity of total RNA were determined by ultraviolet spectrophotometry and agarose gel electrophoresis. cDNA was synthesized by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Samples were loaded in triplicate into 48-well optical plates of the StepOne PCR thermal cycler system, version 2.3 (Applied Biosystems, Lincoln, NE, USA). Each PCR reaction mixture contained 100 ng of cDNA, RealQ PCR 2x Master Mix SYBR Green high ROX® (Amplicon, Stenhuggervej, Denmark), *PI3K* or *NIS* primers (Macrogen, Seoul, South Korea), and RNAase/DNAase-free water (Thermo Scientific). For each sample, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene for normalizing the expression of *PI3K* and *NIS*. The primer sequences are given in Table 1.

qRT-PCR conditions were as follows: incubation (95°C, 10 min), 40 reaction amplifications (30 s at 95ºC, 30 s at 60ºC, and 30 s at 72ºC), and a melting curve (60-95°C). All examinations were performed in triplicate. Serial dilutions (10-fold) of the internal standard were performed to produce a standard curve for each gene in all runs. Correlation coefficients ($r^2 > 0.999$) and efficiency (> 99%) were considered reliable. The Livak equation ($2^{-\Delta\Delta Ct}$) was applied for each of the data points (Livak and Schmittgen, 2001).

Western blotting

Treated cells were lysed in lysis buffer consisting of 50 mM Tris (pH 8.0) (Merck, Darmstadt, Germany), 150 mM NaCl (Merck), 1% Triton X-100 (Sigma-Aldrich), and 10 µg/mL aprotinin (Sigma-Aldrich). Protein concentrations were measured by the Bradford assay (Bio-Rad, Hercules, CA, USA). Total proteins (15 µg), the PageRuler ladder (Thermo Scientific), and healthy human thyrocyte lysate (as a positive control) were loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel and electrophoresed (Mini-PROTEAN Tetra Vertical Electrophoresis Cell, Bio-Rad, Marnes-la-Coquette, France). The bands were transferred (4°C, overnight) onto a Protran Nitrocellulose Transfer Membrane (Sigma-Aldrich). The membrane was blocked (90 min at room temperature) in 5% skimmed milk (Merck) in washing buffer (10 mM Tris–HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween® 20 [Merck]). After washing, it was incubated (1 h at room temperature) with an *NIS* antibody diluted 1:200 (ab17795, Abcam, Cambridge, UK) in 1% bovine serum albumin (Sigma-Aldrich)-washing buffer. Then, the washing steps were repeated and the membrane was incubated (45 min at room temperature) with horseradish peroxidase-conjugated goat anti-mouse IgG (ab97023, Abcam), diluted 1:2000 in 1% bovine serum albumin-washing buffer. Finally, the bands were visualized with 3,3'-diaminobenzidine (Sigma-Aldrich).

Statistical analysis

Graphpad Prism version 6.01 analytic software (Graphpad, San Diego, CA, USA) and the one-way analysis of variance test were used to determine differences in the expression levels of *PI3K* and *NIS* mRNA. A P value

less than 0.0001 was regarded as statistically significant. Data from triplicate experiments are expressed as means ± standard error of the mean.

Results

Verification of CSC purity

CD133-positive CSCs with 92.2, 71.6, and 96.9% purity were isolated from the C643, SW1736, and 8305C cell lines, respectively (Figure 1).

Evaluation of PI3K and NIS mRNA expression in CD133-positive CSCs segregated from ATC cell lines after treatment with LY294002

Analysis of the qRT-PCR data showed that, compared with the level before treatment (control group), the expression of *NIS* mRNA increased in the CD133-positive CSCs separated from C643 (Figure 2A), SW1736 (Figure 2C), and 8305C (Figure 2E) cells after 24 and 48 h of treatment with 5, 10, 20, or 25 μM LY294002. The results also indicated that the expression of PI3K mRNA decreased in the CD133-positive CSCs separated from C643 (Figure 2B), SW1736 (Figure 2D), and 8305C (Figure 2F) cells after incubation with these four concentrations of LY294002. The data are summarized in Table 2.

Increased levels of NIS protein expression in CD133-positive CSCs after treatment with LY294002

To assess the effects of LY294002 on *NIS* protein levels in CD133-positive CSCs, western blotting was performed. The results showed that CD133-positive CSCs isolated from C643, SW1736, and 8305C cells treated with LY294002 were capable of expressing the *NIS* protein compared with pre-treatment. In addition, various LY294002 concentrations caused different changes in expression of the *NIS* protein (Figure 3).

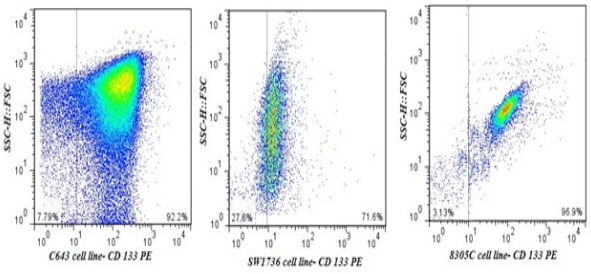

Figure 1. Data Showing CD133-positive Cells Isolated from C643, SW1736, and 8305C Cell Lines with Purity Over 70%.

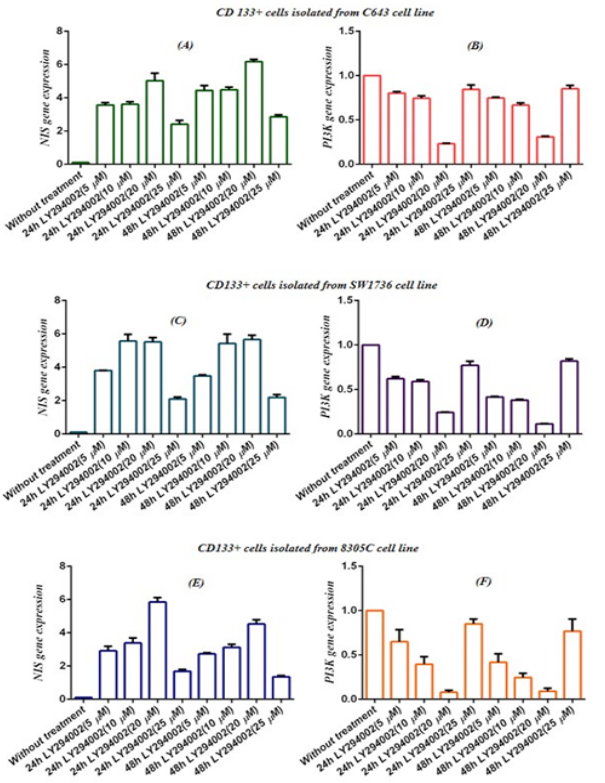

Figure 2. Quantitative Real-Time PCR Analyses Revealed that the Expression *NIS* (A, C, E) and *PI3K* (B, D, F) mRNA was Changed in CD133-positive Cells Isolated from the ATC Cell Lines after Treatment with LY294002. Data are Presented as means ± SEM (n = 3). $P < 0.0001$.

Discussion

In this investigation, we studied anaplastic thyroid CSCs with the CD133 surface marker. CSCs have self-renewal ability and generate diverse cancer cells that are linked to tumor recurrence and drug resistance (Franco et al., 2016). They are identified by the expression of multiple genes (Shimamura et al., 2014) and various surface markers such as CD133 (Grasso et al., 2016; Zhang et al., 2016). These cells are usually present in low numbers (0.5-18%) in tumor tissues or cell lines (Schmohl and Vallera, 2016). Because of their low numbers in ATC cell lines, and for raising the purity of isolated cells, we used the MACS method and repeated the sorting assay for the first flow-through with new MACS columns until reaching a high purity (more than 70%) of CD133-positive CSCs.

Table 1. Accession Numbers, Primer Sequences, and Expected Product Lengths of *PI3K*, *NIS*, and *GAPDH*

Gene	Accession No.	Primer sequence (5′ to 3′)	Product length(bp)
PI3K	NM_006218.2	(F) AACCTCAGGCTTGAAGAG (R) GAAGTGTTAGCATATCTTGC	157
NIS	NM_000453.2	(F) CTATGGCCTCAAGTTCCTCT (R) CGTGGCTACAATGTACTGC	178
GAPDH	NM_002046.5	(F) GCTCTCTGCTCCTCCTGTTC (R) CGACCAAATCCGTTGACTCC	114

F, forward; R, reverse

Table 2. Fold Changes in PI3K and NIS mRNA Levels Using the Livak Method in CD133-Positive Cells Isolated from the ATC Cell Lines after 24 and 48 h Treatment with LY294002.

Cell type	Concentration of LY294002 µM	Time	*PI3K* gene expression Mean ± SEM, n=3, $r^2 > 0.993$, Eff (99.9%)	*NIS* gene expression Mean ± SEM, n=3, $r^2 = 1$, Eff (100%)
CD133pos cells isolated from C643 cell line	5	24 hours	0.80±0.01	3.56±0.11
	10		0.74±0.02	3.61±0.11
	20		0.23±0.0	5.02±0.36
	25		0.84±0.04	2.41±0.18
	5	48 hours	0.74±0.0	4.44±0.23
	10		0.66±0.02	4.48 ±0.13
	20		0.30±0.0	6.17±0.11
	25		0.85±0.03	2.85±0.09
CD133pos cells isolated from SW1736 cell line	5	24 hours	0.62±0.01	3.80±0.0
	10		0.58±0.01	5.57±0.32
	20		0.24±0.0	5.51±0.20
	25		0.77±0.03	2.09±0.10
	5	48 hours	0.41±0.0	3.48±0.05
	10		0.38±0.0	5.42±0.45
	20		0.11±0.0	5.66±0.21
	25		0.82±0.02	2.19±0.14
CD133pos cells isolated from 8305C cell line	5	24 hours	0.65±0.11	2.91±0.21
	10		0.39±0.07	3.38±0.25
	20		0.07±0.01	5.85±0.21
	25		0.85±0.04	1.68±0.09
	5	48 hours	0.41±0.07	2.72±0.05
	10		0.24±0.03	3.12±0.14
	20		0.09±0.02	4.52±0.21
	25		0.76±0.11	1.34±0.06

Analysis of the qRT-PCR data revealed that various concentrations (5, 10, 20, and 25 µM) and times (24 and 48 h) of LY294002 treatment had different effects on *PI3K* and *NIS* mRNA levels. We found that 20 µM of this inhibitor was most effective at raising and diminishing *NIS* and *PI3K* mRNA levels, respectively. LY294002 at 25 µM had the least influence on the expression of *NIS* and *PI3K*. This indicates that an elevated concentration of the PI3K inhibitor limited its action on *NIS* expression. We believe that other factors may be involved in this mechanism and should be studied in the future. In addition, although all cells isolated from the three distinct ATC cell lines (C643, SW1736, and 8305C) were treated under identical conditions, the fold inductions of *NIS* were not the same. These data illustrate that intensifying activity of the *PI3K*/Akt pathway in CSCs plays a dominant role in activating *NIS*. Our findings are consistent with previous studies (Liu et al., 2012; Serrano-Nascimento et al., 2014).

Recent studies revealed that PI3K activation can modify *NIS* on the cell surface and reduce all-trans retinoic acid and hydrocortisone-influenced glycosylated *NIS* protein expression, and *NIS*-mediated RAI uptake (Knostman et al., 2004; Li et al., 2006; Ohashi et al., 2009; Wu et al., 2005). Based on our PI3K expression findings, *NIS* protein expression was assessed in the treated CSCs.

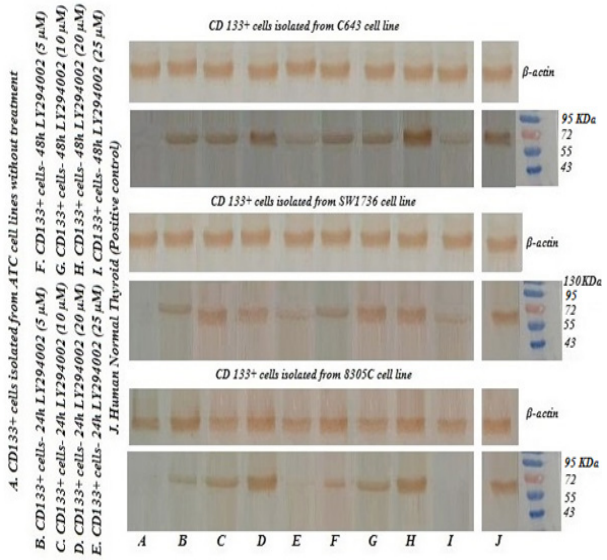

Figure 3. Western Blotting Shows that, Before Treatment (A), CD133-positive Cells Isolated from ATC Cell Lines Expressed Low Levels of the *NIS* Protein. Various LY294002 Concentrations Caused Different Changes in Expression of the NIS Protein (B-I). Normal Human Thyrocyte Lysate (J) and β-actin were Used as the Positive Control and for Normalization of Protein Expression, Respectively.

We found that CD133-positive CSCs isolated from the 8305C cell line and treated with 25 µM LY294002 for 24 and 48 h did not express increased *NIS* protein even though the mRNA levels were increased 1.68 ± 0.09- and 1.34 ± 0.06-fold at 24 and 48 h, respectively. The mRNA levels of other CD133-positive CSCs that expressed levels of this protein, were increased more than 2-fold. This indicates that expression of the *NIS* protein is linked to its mRNA levels. Other factors may also be involved in the process of protein expression that need to be clarified in future studies.

Overall, our data indicate that expression of the *NIS* protein in CSCs is very low and thus not a major factor in the iodide uptake process. However, we believe that focusing on CSCs in a targeted therapy manner is an effective way to create successful treatments for ATC patients. Our study shows that these cells express *NIS* mRNA and protein as a thyroid differentiation marker. It may be possible to eradicate these cells with several strategies such as the use of toxins (Waldron et al., 2014; Waldron and Vallera, 2013), immunotoxins (Ohlfest et al., 2013), monospecific anti-CSCs antibodies (Damek-Poprawa et al., 2011), anti-CSC conjugated nanoparticles (Swaminathan et al., 2013), anti-CSCs bi-specific antibodies (Huang et al., 2013), trispecific natural killer engagers (Schmohl et al., 2016; Waldmann, 2014), and aptamers (Shigdar et al., 2013).

In conclusions, our results reveal that CD133-positive CSCs found in ATC cell lines do not have *NIS* gene/protein expression as a major factor for RAI therapy. Thus, we believe that the presence of CSCs in tumor tissue, particularly ATC, is a likely reason for the failure of RAI therapy. Molecular CSC targeted therapy could be a treatment of choice to improve RAI therapy. Our data show that by targeting these cells with a PI3K inhibitor, we

could induce expression of *NIS* mRNA and protein. This may improve the efficacy of RAI therapy in aggressive cancers like ATC.

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Conflict of interest

The authors declare no conflict of interest.

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