Targeting the BRAF Signaling Pathway in CD133pos Cancer Stem Cells of Anaplastic Thyroid Carcinoma

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Abstract

Background: Cancer stem cells (CSCs) with a self-renewal ability in tumor cell populations, execute a pivotal function in tumorigenesis, regression, and metastasis of malignant cancers such as anaplastic thyroid carcinoma (ATC). Materials and Methods: In this study, we isolated CSCs subpopulation with CD133 surface marker from three ATC cell lines by magnetic cell sorting assay. After confirming the segregation by the flow cytometry method, BRAF and sodium-iodide symporter (NIS) genes were investigated in them before and after incubation with BRAF inhibitor. Also, we evaluated the NIS protein expression and localization. Results: Established upon q-RT PCR data, when compared to human normal thyrocytes, the BRAFV600E gene was over-expressed in CD133pos cells (>1705.99 ± 55.55 fold, Mean ± SEM, n=3, P-value<0.05), whilst the expression of NIS gene was very restricted (< 0.0008 ± 5.43 fold, Mean ± SEM, n=3, P-value<0.05) in them. Also, our results showed that BRAF inhibition affected NIS protein expression and localization. Conclusions: Current study showed that the differentiate genes/proteins expression can be induced in the CSCs via focus on signal transduction pathways and targeting their molecules, that are involved in expression of these genes/proteins. Therefore, attention to targeting CSCs along with routine thyroid cancer therapy, can help to ATC treatment.

Keywords: Anaplastic thyroid carcinoma- positive CD133 cancer stem cells- BRAF inhibition- sodium-iodide symporter

Asian Pac J Cancer Prev, 20 (5), 1353-1360

Introduction

A rare and malignant cancer cells with a self-renewal ability in tumor cell populations, are explained as cancer stem cells (CSCs). They play a pivotal role in tumor launching, tumor size, regression, and metastasis to the thyroid gland and other encircling tissues (Li et al., 2013). Usually, they are recognized by specific surface markers such as CD133. This antigen, with 865 amino acids, formed five trans-membrane glycoproteins that are localized on plasma membrane of the CSCs and generated the CD133pos cells with tumorigenic potential (Liu et al., 2016a; Bozorg-Ghalati et al., 2017a). In recent years, researchers have demonstrated that thyroid CSCs, as undifferentiated cells with clonogenic capacity, are excessively resistant to radio/chemo therapy; therefore, caused the thyroid cancer to relapse (Guo et al., 2014; Ke et al., 2013; Bozorg-Ghalati et al., 2017b). Indeed, with stress to their special characteristics, they play a major role in determined opposition to routine therapies of mortal malignant cancers, such as anaplastic thyroid carcinoma (ATC) (Zito et al., 2008; Vinogradov et al., 2012).

ATC is thinly scattered, but it is the most aggressive cancer with quelled survival rates of all thyroid cancers. Its tumor cells have an undifferentiated phenotype, irregular large nuclei, and a superfluous nuclear/cytoplasmic (N/C) ratio. ATC is frequently a lethal neoplasia and usually is resistant to chemo/radioiodine therapy as common thyroid cancer therapy (Landa et al., 2016; Bozorg-Ghalati et al., 2017c). Thyroid-specific differentiation markers such as thyroglobulin (Tg), thyrotropin receptor (TSHR), sodium-iodide symporter (NIS), and thyroid peroxidase (TPO) are members of the thyroid hormones production system. These agents, especially the NIS protein, play a major role in the radioiodine uptake and effective radioactive iodine therapy (Tang et al., 2014).

Based on the data of associated studies, various factors such as somatic gene mutation, can be engaged in gene and protein expression of these agents and also can inhibit their functions (Bozorg-Ghalati et al., 2015; 2017d). BRAF (B-Rapidly Accelerated Fibroblast) as a serine/threonine-protein kinase, is a member of the...
mitogen-activated protein kinase (MAPK) pathway. Its gene mutations, like $BRAF^{V600E}$, with different ways, such as the intensive activation of TGFβ/Smad signaling, histone deacetylation of NIS promoter, and the regulation of DNA methyl transferase 1, can be effected on the NIS gene expression and function (Bozorg-Ghalati et al., 2016; Choi et al., 2014; Zhang et al., 2014; Riesco-Eizaguirre et al., 2009).

Nowadays, elimination or differentiation of CSCs due to targeting them, is new insight for treatment of aggressive carcinomas such as ATC (Vicari et al., 2016). Indeed, target therapy and focusing on the CSCs, as potential targets, are controversial debate for cancers therapy (Madka et al., 2011). Separate previous studies were explained the frequency of BRAF mutation (Lim et al., 2016; Rosove et al., 2013), and the role of CSCs in thyroid cancers (Jung et al., 2015; Decaussin-Petrucci et al., 2015).

Nevertheless, the relationship among mutant BRAF and thyroid CSCs is largely unknown. Thus far, only very limited data are avail regarding thyroid CSCs, their molecular and signaling pathway information’s, and particularly unpublished data about their BRAF and NIS gene levels. Therefore, we managed this study to emphasize on the BRAF signal transduction pathway in CD133pos cells existing in ATC cell lines. Also, we investigated thoroughly the expression levels of $BRAF$ and NIS genes in these cells and appraised the BRAF inhibition effects on their NIS gene/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 performed in accordance with international policies established by the Declaration of Helsinki.

Anaplastic Thyroid Carcinoma cell lines and cell culture

The ATC cell line (8305C) was bought from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). The cells were cultured at 37°C in DMEM-Glutamax (Biowest, Nuaillé, France). Two another ATC cell lines (SW1736 and C643) were benevolently provided by Dr. Vahid Haghpanah (Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences, Tehran, Iran). We cultivated them at 37°C, 5% CO2, in RPMI 1640 GlutaMAX™ medium (Biowest, Nuaillé, France). All media were supplied with 10% inactivated fetal bovine serum (Gibco™, EU Approved South American), 1% pen-strep (Biowest, Nuaillé, France) and 1% non-essential amino acids (Biowest).

Magnetic-activated cell sorting (MACS) assay

Cells with CD133 surface marker were isolated from the three above ATC cell lines by MACS method. The human CD133 Micro Bead Kit-Tumor Tissue (Miltenyi Biotec, Bergisch Gladbach, Germany) was used and the method was performed according to the manufacturers’ protocol. Briefly, subsequent to culture the cell lines; they were harvested by trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged (300×g, 10 min). The cell pellets were resuspended in 60 μL of MACS buffer (Miltenyi Biotec), 20 μL of FcR blocking reagent and 20 μL of CD133 micro beads per 107 total cells. After incubation for 15 min at 4°C under slow and continuous rotation, the cells were washed, centrifuged (300×g, 10 min), and resuspended in 500 μL of MACS buffer. The cell suspensions were injected separately onto the LS column (Miltenyi Biotec). Then, the flow-through came together and washed the LS column. Finally, after adding 5 mL MACS buffer, the magnetically marked CD133 cells were flushed out by sturdily inserting the piston into the column.

Flow cytometry

According to the Miltenyi Biotec company protocol, we added 10 μL of CD133 antibody (Miltenyi Biotec) to 100 μL of cell suspension. This was mixed well and incubated (4°C, 10 min). Subsequently, by adding 1-2 mL of MACS buffer, the cells were washed, centrifuged (300×g, 10 min), and the cell pellets were resuspended in buffer and analyses were performed by flow cytometry (FACS Calibur; BD Biosciences, Franklin Lakes, NJ, USA).

Treatment

The CD133pos cells were treated with 5μg/mL bovine thyroid-stimulating hormone (Sigma-Aldrich) and separate Selumetinib (Chemietek, Indianapolis, IN, USA) concentrations (10, 15, 20, or 25nM) for 24 and 48 hours, respectively.

RNA isolation and cDNA synthesis

The extraction of the total RNA was performed by using the YTA Total RNA Extraction Mini Kit (Yekta Tajhiz Azma, Tehran, Iran). After determining the purity, quantity and integrity of the total RNA, the cDNA was synthesized by the Revert Aid First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific, Waltham, MA, USA).

Quantitative Real-time PCR assay

A real-time PCR was performed with the Step One PCR thermal cycler system version 2.3 (Applied BioSystems, Lincoln, NE, USA). Each sample mixture contained: 100 ng of cDNA, 10 pM of $BRAF$ and NIS gene primers (Macrogen, Seoul, South Korea) (Table 1), RNAase and DNAase free water (Thermo Scientific), and Real Q PCR 2x Master Mix SYBR Green high ROX™ (Amplicon, Stenhuggervej, Denmark) for the detection of gene expression analysis. In each run, the samples were loaded in triplicate on a 48-well optical. For each gene, in all runs, a set of tenfold serial dilutions of the internal standard was used to generate a standard curve with reliable correlation coefficients ($r^2=0.999$). Forty reaction amplification cycles embracing: 30 s at 95°C, 30 s at 60°C and 30 s at 72°C for NIS gene, and 15 s at 95°C, 60 s at 60°C for BRAF gene were activated after 10 min incubation at 95°C. A melting curve (60-95°C) analysis was performed for each run.
and sample. For gene normalization, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control.

**Western blot**

After treatment, we used a lysis buffer, which was comprised 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), for cell lysis. Protein concentrations were measured by Bradford assay (Bio-Rad, Hercules, CA, USA). Fifteen μg of total proteins, and page ruler ladder (Thermo Scientific) were loaded to the 10% SDS-PAGE gel (Mini-PROTEAN Tetra Vertical Electrophoresis Cell, Bio-Rad, Marnes-la-Coquette, France). A healthy human thyroid tissue lysate was used as a positive control. After terminating the running assay, the bands were transferred to the Protran Nitrocellulose Transfer Membrane (Sigma-Aldrich) at 4°C overnight. The membrane was blocked (90 min, at RT) with 5% skimmed milk (Merck) in washing buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween® 20 [Merck]). Afterwards, it was washed well and incubated (1h, RT) with an NIS antibody (ab17795, Abcam, Cambridge, UK), which was 1:200 diluted in 1% BSA-washing buffer. The washing steps were repeated and the membrane incubated (45 min, RT) in 1.2000 diluted horseradish peroxidase conjugated goat anti-mouse IgG (ab97023, Abcam), in 1% BSA-washing buffer. Finally, the 3, 3'-diaminobenzidine (DAB) (Sigma-Aldrich) was used for the bands’ visualization.

**Immunocytochemistry (ICC)**

Following treatment, the cells were fixed with -20°C pre-chilled absolute methanol (Merck), washed by PBS (Sigma-Aldrich), and incubated (10 min, RT) in PBS-0.1% Triton X-100. Then, they were incubated (30 min, RT) in 10% diluted goat serum (Biowest), with PBS-5% BSA (Sigma-Aldrich), and 0.5% Tween® 20. The solution was discarded and the cells were incubated with 1:100 diluted NIS antibody in a mixture solution (PBS, 1% BSA, 0.1% Tween® 20) in a humidity chamber (overnight, 4°C). After washing three times with PBS, the cells were incubated (1h, RT) in the dark with goat anti-mouse IgG (1:200 diluted in PBS-1% BSA). DAB and hematoxylin (Sigma-Aldrich) were used for detection and counterstain, respectively. Eventually, we identified the specific location of NIS protein by invert microscope (Olympus, Tokyo, Japan).

**Statistical analysis**

It was noticeable that all experiments were done in duplicate manner. Dependent upon the operated samples, the data of real time-PCR were analyzed with the Livak et al., (2001) or Pfaffl (2001) methods. The One-way ANOVA test and the Graph Pad Prism version 6.01 software (Graphpad, San Diego, CA, USA) were used for the statistical analysis and showing the fold changes of BRAF and NIS mRNA expression levels. The analyzed data declared as Mean ± SEM of three independent tests. A P-value < 0.05 was considered statistically significant.

**Results**

**Confirming the CD133pos cells segregation**

The flow cytometry assay confirmed the purity and sorting of the CD133™ cells derived from ATC cell lines (Figure 1). Despite a low rate of CD133 cells in the original cell lines (Figure 1, A-C), they were isolated with 95.3%, 90.3%, and 89.0% purity from C643, SW1736, and 8305C cell lines, respectively (Figure 1, D-F).

![Figure 1](image_url)

Figure 1. Results of Flow Cytometry Method. Flow cytometry assay confirmed the purity and sorting of the CD133™ cells derived from ATC cell lines (C643, SW1736 and 8305C, respectively) after using MACS method (D-F) compared to before isolation (A-C).
Survey of the BRAF and NIS genes expression in ATC cell lines and CD133\textsuperscript{pos} cells compared to the human normal thyrocytes

We compared the BRAF and NIS genes expression between ATC cell lines, CD133\textsuperscript{pos} cells and human normal thyrocytes. The results revealed that the CD133\textsuperscript{pos} cells have huge expression level of the BRAF gene compared to human normal thyrocytes and ATC cell lines (Figure 2). This gene was expressed at 1,705.99 ± 55.55, 7,265.85 ± 511.91, and 6543.95 ± 136.15 levels in the CD133\textsuperscript{pos} cells that were isolated from C643, SW1736, and 8305C cell lines, respectively. In C643, SW1736, and 8305C cell lines, the expression levels of this gene were 509.87 ± 52.28, 2,461.43 ± 310.91, and 1,303.56 ± 148.15 folds, respectively.

As displayed in Figure 3, the NIS mRNA levels are very low in C643 (B, 0.29 ± 0.04), SW1736 (D, 0.29 ± 0.11), and 8305C (F, 0.35 ± 0.03) cell lines compared to human normal thyrocytes (A). It is astonishing that this gene has tiny level in CD133\textsuperscript{pos} cells segregated from C643 (C, 0.0003 ± 2.71), SW1736 (E, 0.0002 ± 0.71), and 8305C (G, 0.0008 ± 5.43) cell lines, respectively.

Table 1. Accession Numbers, and Primer Sequences of BRAF, NIS, and GAPDH

| Gene | Accession No | Primer Sequence (5’ to 3’) |
|------|--------------|----------------------------|
| BRAF | NM_004333.4  | (F)CAGTCTAAAAGGAACACTGTAG (R)CAGGAATAATCGTCTCCA |
| NIS  | NM_000453.2  | (F)CTATGGCCCTAAGITCCTCT (R)CGTGCTACAATGTACTGC |
| GAPDH| NM-002046.5  | (F)GGCTTCTGGTCCTCCTGCTT (R)CGACCAAATCCGTTGACTCC |

Figure 2. Evaluate the BRAF Gene Expression in Human Normal Thyrocytes, ATC Cell Lines and the CD133\textsuperscript{pos} Cells Isolated from them. Data (Mean ± SEM, n=3) showed that despite this gene has not any level expression in human normal thyrocytes (A), has a huge expression level in the CD133\textsuperscript{pos} cells isolated from C643 (C, 1705.99 ± 55.55), SW1736 (E, 7265.85 ± 511.91), and 8305C (G, 6543.95 ± 136.15) cell lines. In C643, SW1736, and 8305C cell lines, expression levels of this gene were 509.87 ± 52.28, 2461.43 ± 310.91, and 1303.56 ± 148.15, respectively. GAPDH housekeeping gene was used for mRNAs levels normalization. *P<0.05 by One-way ANOVA test.

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Assessment of the BRAF and NIS genes expression in CD133\textsuperscript{pos} cells segregated from ATC cell lines after treatment with Selumetinib

We evaluated the effects of Selumetinib on BRAF and NIS genes expression in CD133\textsuperscript{pos} cells. As presented in Figure 4, fold inductions of the BRAF and NIS mRNAs levels were reduced and increased in the CD133\textsuperscript{pos} cells after treatment, respectively.
Change in ability of the NIS protein expression in the CD133<sup>pos</sup> cells after BRAF inhibition

The results of the Western blotting showed that different concentrations and times of Selumetinib, caused the CD133<sup>pos</sup> cells gain the ability to express a 69 kDa NIS protein. This ability tended to be in line with the cells’ corresponding quantitative real-time PCR data (Figure 5).

BRAF inhibitor can be engaged in the NIS protein localization

Based on the real-time PCR data, the NIS gene was highly expressed in CD133<sup>pos</sup> cells after 24 and 48 hours treatment with 20 and 10 nM of Selumetinib, respectively. The ICC results showed that after incubation with these drug concentrations, compared to previous treatments, the NIS protein was presented in cytoplasm and plasma membrane of some CD133<sup>pos</sup> cells (Figure 6).

Discussion

In the present study, with considering the major and critical roles of thyroid CSCs (Nagayama et al., 2016), we investigated the NIS mRNA level and BRAF<sub>V600E</sub> mutation in CD133<sup>pos</sup> cells existing in ATC cell lines. For this purpose, we separated these cells from three ATC cell lines (C643, SW1736, and 8305C) by the MACS method. The first flow cytometry results showed that the rate of these cells was approximately 9-13% in our ATC cell lines. Despite their low rate, after several separations and using a new MACS LS column, they isolated with a final purity of more than 80%. However, these results were related to the start time of the isolation, and the grade of the CD133<sup>pos</sup>...
We analyzed the expression of \textit{NIS} gene in CD133\textsuperscript{pos} cells and their original ATC cell lines. By contrasting the expression of this gene in human normal thyrocytes, we discovered that it was low-expressed in ATC cell lines, while its expression in CD133\textsuperscript{pos} cells was very restricted. The \textit{NIS} gene/protein expression is consequential for radioiodine uptake and effective radioactive iodine therapy (Morgan et al., 2016).

Our results provide new insight about mechanism of radioiodine-resistance of CD133\textsuperscript{pos} thyroid CSCs. Prior studies on CD133\textsuperscript{pos} glioma CSCs have shown that because of different reasons such as diminish rates of apoptosis signaling, elevate activation of ATM, Rad17, Chk1, and Chk2 (members of DNA damage repair system), and reduce levels of reactive oxygen species, these cells are resist to radiation therapies (Eyler et al., 2008; Mita et al., 2008; Diehn et al., 2009).

In our findings, the \textit{NIS} gene inexpression in CD133\textsuperscript{pos} cells is a significant point. Because they have not any \textit{NIS} gene expression, therefore they cannot uptake radioactive iodine. Scilicet, the CD133\textsuperscript{pos} cells are inside the ATC cell lines, but the \textit{BRAF}\textsuperscript{V600E} expression levels in ATC cell lines are lower than their separated CD133\textsuperscript{pos} cells. Besides, the CD133\textsuperscript{pos} cells isolated from SW1736 and 8305C cell lines have a salient \textit{BRAF} expression rather than another CD133\textsuperscript{pos} cells separated from the C643 cell line. We believed that these points should be examined in future studies.

We hypothesized that, with BRAF inhibition, the CD133\textsuperscript{pos} cells are capable of expressing the \textit{NIS} gene and protein. To this purpose, we used \textit{Selumetinib} (AZD6244) in various concentrations at different times. We evaluated the \textit{BRAF}\textsuperscript{V600E} and \textit{NIS} mRNA levels in treated CD133\textsuperscript{pos} cells by using quantitative real-time PCR assay. However, the mRNA levels of these genes in the CD133\textsuperscript{pos} cells isolated from three ATC cell lines, were not exactly the same. From a general view and established upon our real-time PCR analysis, \textit{BRAF}\textsuperscript{V600E} gene reduction occurred in CD133\textsuperscript{pos} cells after this treatment. Also, these cells were able to express the \textit{NIS} gene. Preceding studies have shown that BRAF mutation can be involved in the \textit{NIS} gene expression of papillary thyroid cancer (Yazgan et al., 2016; Barollo et al., 2010; Dong et al., 2016).

Furthermore, our results showed that the \textit{NIS} gene expression level in the CD133\textsuperscript{pos} thyroid CSCs. The role of CD133\textsuperscript{pos} cells with their huge expression of \textit{BRAF} gene in ATC recurrence is highlighted. Based on these observations, it seems that the CD133\textsuperscript{pos} cells are the main source of \textit{BRAF}\textsuperscript{V600E} mutation in ATC cell lines. However, remark on its expression level in CD133\textsuperscript{pos} cells, which is higher than their original ATC cell lines, is the big subject of controversy. Scilicet, the CD133\textsuperscript{pos} cells are inside the ATC cell lines, but the \textit{BRAF}\textsuperscript{V600E} expression levels in ATC cell lines are lower than their separated CD133\textsuperscript{pos} cells. Besides, the CD133\textsuperscript{pos} cells isolated from SW1736 and 8305C cell lines have a salient \textit{BRAF} expression rather than another CD133\textsuperscript{pos} cells separated from the C643 cell line. We believed that these points should be examined in future studies.

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gene expression correlates with BRAF gene mutation particularly in CD133pos CSCs of ATC. In addition, our results suggested that the Selumetinib can be induced the NIS gene expression as a thyrocyte differentiation marker in undifferentiated cells like CD133pos thyroid CSCs.

We focused on the CD133pos CSCs to investigate the NIS protein expression ability. Our Western blotting results revealed that the value of BRAFV600E gene reduction had an effect on the NIS protein expression. In addition, the amount of NIS mRNA is connected to its protein expression. Scilitic, if the NIS mRNA level was raised to two folds, we could detect its protein.

In our study, the CD133pos cells have the most NIS gene expression (≈ 3-6 fold) after 24 and 48 hours incubation with 20 and 10 nM Selumetinib concentrations, respectively. On the basis of these results, we analyzed the location of NIS protein by the ICC assay. Until now, we do not receive any information about the NIS protein localization of CD133pos CSCs. Our results disclosed that the NIS protein exists in the cytoplasm and plasma membrane, in comparison to the previous treatments. Hence, the mutant BRAF gene is highlighted as one reason for interfering in the NIS protein localization. Accordingly, the CD133pos CSCs sufficient capability and ability to uptake radioiodine is significant point and must be evaluated in future examinations.

In the present examination it has been elucidated that NIS protein affected by the BRAFV600E gene in CD133pos cells. However, the affected BRAFV600E gene on NIS gene/protein expression (Bastos et al., 2015) is one agent of several factors that cause a loss of NIS expression (Lyckesvärd et al., 2016).

In conclusion, in all types of thyroid cancers, ATC is an undifferentiated malignancy with an indigent prospect. Although, ATC is resistant to customary thyroid cancer therapy, but with targeting the thyroid CSCs might be solved the radioiodine resistance of ATC tumor cells. Current study showed that the differentiate genes/proteins expression can be induced in the CSCs via focus on signal transduction pathways and targeting their molecules, that are involved in expression of these genes/proteins. Therefore, attention to targeting CSCs along with routine thyroid cancer therapy, can help to ATC treatment.

**Conflicts of interest**

The authors made no conflict of interest.

**Acknowledgments**

The authors wish to thank the Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, IRAN, for the financial support. The present study was done in fulfillment of the requirements for PhD degree awarded to F. Bozorg-Ghalati [Grant number: 6066]. Thanks also go to Dr Reza Ranjbaran and Dr Iraj Mohammad pour for their technical assistances.

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