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Do cell surface trafficking impairments account for variable cell surface sodium iodide symporter levels in breast cancer?

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Abstract

The Na⁺/I⁻ symporter (NIS) is a transmembrane glycoprotein that mediates iodide uptake into thyroid follicular cells and serves as the molecular basis of radioiodine imaging and therapy for thyroid cancer patients. The finding that NIS protein is present in 80-90% of breast tumors suggests that breast cancer patients may also benefit from NIS-mediated radionuclide imaging and targeted therapy. However, only 17-25% of NIS-positive breast tumors have detectable radionuclide uptake activity. The discrepancy between NIS expression and radionuclide uptake activity is most likely contributed by variable cell surface NIS protein levels. Apart from the prevalent view that NIS cell surface trafficking impairments account for the variability, our current study proposes that differential levels of NIS expression may also account for variable cell surface NIS levels among breast tumors. We address the need to confirm the identity of intracellular NIS staining to reveal the mechanisms underlying variable cell surface NIS levels. In addition, we warrant a quantitative correlation between cell surface NIS levels and radionuclide uptake activity in patients such that the cell surface NIS levels required for radionuclide imaging can be defined and the defects impairing NIS activity can be recognized.

Keywords

Breast cancer; glycoprotein; iodide uptake; radionuclide imaging and therapy; sodium iodide symporter (NIS)

Introduction

The Na⁺/I⁻ symporter (NIS) is a transmembrane glycoprotein most commonly known for its role as the mediator of active iodide uptake in thyroid follicular cells. NIS transports I⁻ from the bloodstream against its concentration gradient into thyroid follicular cells for the biosynthesis of thyroid hormones triiodothyronine (T3) and thyroxine (T4). In addition to its role in normal thyroid physiology, NIS has been exploited as the cornerstone for imaging and

targeted radioiodine therapy to ablate residual differentiated thyroid carcinomas and metastases after total thyroidectomy [1].

NIS expression is induced in the breast during late pregnancy and lactation in order to accumulate iodide for the nursing infant to synthesize its own thyroid hormones [2-3]. Over forty years ago radioactive iodide uptake activity was also detected in malignant breast tumors, suggesting the possible role of NIS-mediated radionuclide imaging for diagnosis and targeted therapy of breast cancer [4]. However, it was not until after NIS cDNA was cloned [5-6] that NIS mRNA [7-9] and protein expression [10-15] could be examined in breast cancer. To date, NIS expression/function in human breast cancer has been detected by RT-PCR [7-9], RNase protection assay [12], Western blot [12], scintigraphy [8,14], and most commonly immunohistochemistry [10-15]. The majority of studies showed that NIS is expressed in 80%-90% of breast cancers with diverse histological and molecular subtypes. However, only 17%-25% of NIS-positive breast tumors demonstrate detectable radionuclide uptake activity [8,14] and the levels of radionuclide accumulation may not be sufficient for therapeutic purposes.

The objective of this study is to identify possible mechanisms underlying this discrepancy between NIS expression and radionuclide uptake activity. Based on our literature review and the results of our own studies, the discrepancy is most likely contributed by differential NIS levels at the cell surface and/or the heterogeneity of NIS-positive cells within breast tumors. The prevalent view believes that the variable cell surface NIS levels among breast tumors are mainly contributed by NIS cell surface trafficking impairments rather than differences in NIS expression, as current literature reports that many tumors had predominantly intracellular NIS staining. However, our experimental data suggests that some of the reported intracellular NIS staining may be contributed by cross-reactivity of antibodies, and thus the prevalence of NIS cell surface trafficking defects in breast cancer may be overestimated. We propose several strategies to confirm the identity of intracellular NIS staining and warrant a comprehensive study to define the cell surface NIS levels as well as the number of NIS-positive cells required for detectable radionuclide uptake activity *in vivo*.

Materials and Methods

Literature review

Published primary research articles that examined NIS protein levels in human breast tumor samples were included in the survey. Six articles examined NIS protein in breast cancer by immunohistochemistry [10-15] of which one performed Western blot analysis [12]. Publications based solely on cell lines, animal models or review articles were excluded from the study.

Tissue microarrays

Nine tissue microarrays composed of 210 breast cancers were examined. Three cores of 1 mm in diameter corresponding to different regions of each tumor were included in the array. Of the 210 cases, 192 were evaluable for this study.

NIS antibodies

Affinity purified #331 and #442 as well as non-purified #836 polyclonal human NIS antibodies were custom-generated against the myelin basic protein (MBP)-human NIS fusion protein (amino acids 468-643 of human NIS) (Sigma Genesys, The Woodlands, TX). FP-13 monoclonal human NIS antibody was raised against the same antigen [16]. VJ1 NIS monoclonal mouse antibody recognizing the extracellular loop of human NIS was kindly provided by Dr. Sabine Costagliola, Institute of Interdisciplinary Research, Free University of

Brussels, Belgium [17]. The PA716 polyclonal rat NIS antibody raised against the rat NIS peptide (amino acids 603-618) was kindly provided by Dr. Bernard Rousset, Institut National de la Santé et de la Recherche Médicale, Lyon, France [18].

Immunohistochemical staining

Paraffin-embedded tissue sections or tissue microarrays of 4 µm thickness were heated in a 60°C oven for 1 hour, cooled to room temperature, deparaffinized with xylene and rehydrated with graded ethanol solutions. Endogenous peroxidase was blocked by incubating tissues with 3% hydrogen peroxide in water for 5 minutes. Antigen retrieval was then performed using steaming Target Retrieval Solution (Dako Cytomation, Denmark) with a pH of 6.1 for 30 minutes. The following immunostaining procedures were performed by the Dako Autostainer (Dako Cytomation, Denmark). Briefly, tissues were incubated with either #442 (1:25), #836 (1:300) or VJ1 (1:10) primary human NIS antibodies for one hour. Tissues were then rinsed in Tris-Buffered Saline Tween-20 (TBST) buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% Tween-20) and blocked for endogenous biotin using the Biotin Blocking System (Dako Cytomation, Denmark). A Labeled Streptavidin-Biotin (LSAB) Complex Detection System (Dako Cytomation, Denmark) was used for signal amplification per the manufacturer's protocol. Tissues were washed and incubated with diaminobenzidine (DAB) chromagen (Dako Cytomation, Denmark) for 5 minutes for visualization. Tissues were rinsed in distilled water, counterstained with hematoxylin, rinsed, immersed in 1% NH₃OH solution, rinsed, dehydrated through graded ethanol solutions and dipped in xylene. Slides were mounted with Micromount solution (Surgipath Medical Industries, Richmond, IL).

Interpretation of immunohistochemical staining

The level of cell surface NIS in each case was scored on a scale of 0, 1+, 2+, 3+, using criteria analogous to the evaluation of Her-2/neu staining. Absent staining was scored as 0; weak cell surface staining was scored as 1+; weak to moderate cell surface staining was scored as 2+; and strong cell surface NIS staining was scored as 3+. Cytoplasmic staining was also scored on a scale of 0, 1+, 2+ and 3+. Cases with scores of 2+ and 3+ were considered positive for intracellular staining.

Cell culture

MCF-10A breast epithelial cells were cultured in a 1:1 solution of DMEM and Ham's F-12 media (Gibco), 10% horse serum, 1% penicillin/streptomycin and 1% hormone mixture (20 ng/mL EGF, 10 µg/mL bovine insulin, 0.2 mM sodium bicarbonate and 0.5 µg/mL hydrocortisone). MCF-7 breast cancer cells were cultured in a 1:1 solution of DMEM and Ham's F12 medium, 10% FBS and 1% penicillin/streptomycin. MB-231 and SK-Br-3 breast cancer cell lines were each cultured in RPMI media with 10% FBS and 1% penicillin/streptomycin. FRTL-5 immortalized rat thyroid cells were cultured in Coon's modified F-12 media with 5% calf serum, 2 mM glutamine, 1% penicillin/streptomycin, 10 mM NaHCO₃, and 6H hormone mixture (1 mU/mL bovine TSH, 10 µg/mL bovine insulin, 10 nM hydrocortisone, 10 ng/mL somatostatin, 5 µg/mL transferrin and 2 ng/mL L-glycyl-histidyl-lysine). All cells were maintained in a 37°C incubator with 5% CO₂.

Western blot analysis and deglycosylation

Western blot analysis was performed as described [20] with the following modifications. Protein extracts of 100 µg were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. The primary NIS antibodies used were: #331 human NIS (1:1000), FP-13 human NIS (1:5000) or PA716 rat NIS antibody (1:1500). HRP-conjugated anti-rabbit IgG secondary antibody (1:4000) was used for #331 and PA716 polyclonal NIS antibodies and anti-mouse IgG secondary antibody (1:4000) was used for FP-13 monoclonal NIS antibody.

For deglycosylation, 200 µg of protein were denatured with buffer (0.5% SDS and 1% B-mercaptoethanol) and incubated with 500 units of Peptide N-Glycosidase F (PNGase-F) (New England Biolabs, Ipswich, MA). The deglycosylation reaction was enhanced by the addition of reducing sample buffer.

Radioactive iodide uptake assay (RAIU)

RAIU assay was performed as described in Knostman et al. [19] with modifications. Counts per minute (CPM) were normalized to cell number. NIS-mediated RAIU was confirmed by comparing with the addition of NaClO₄ NIS inhibitor in parallel experiments. Fisher Rat thyroid (FRTL-5) cells, an immortalized rat thyroid follicular cell line, served as a positive control.

Results and Discussion

Variable cell surface NIS protein levels may account for the discrepancy between NIS levels and radionuclide uptake activity

To examine whether the observed disparity in NIS expression and radionuclide uptake activity is due to variable cell surface NIS levels, we evaluated cell surface NIS protein levels of 192 invasive ductal breast carcinomas on a scale of 0-3+ according to intensity of plasma membrane staining. Among these samples, 15 (8%) were scored 3+, 40 (21%) were 2+, 83 (43%) were 1+, and 54 (28%) were assigned a score of 0 for cell surface NIS immunostaining with #442 human NIS antibody (Fig. 1). Accordingly, only a fraction of NIS-positive tumors, 8% (3+) to 29% (2-3+), have cell surface NIS levels that may be sufficient to confer detectable radionuclide uptake activity *in vivo*.

It is important to note that most studies included in the literature scored NIS positivity based on cell surface and/or intracellular NIS staining without distinguishing the percentage of tumors that had prominent cell surface NIS from tumors that had prominent intracellular NIS. Our previous study [15] was the only study to report that 27% of NIS-positive tumors (21% of total tumors examined) had cell surface NIS protein. Furthermore, all published studies, including our own, utilized NIS antibodies generated against the C-terminal intracellular region of NIS without confirming with additional antibodies against epitopes in different regions.

As summarized in Table 1, previous studies have detected cell surface and/or predominantly intracellular NIS protein in 80-90% of breast tumors by immunohistochemistry [10-13,15]. In the two studies [11,13] that distinguished strongly positive NIS staining, only 30-40% of breast tumors were scored as strongly positive. These results are in agreement with our study showing only 29% of breast tumors with scores of 2+ or above. While the overall percentage of NIS-positive tumors does not seem to be different between ductal carcinoma *in situ* and invasive carcinoma [10,13], the percentage of NIS-positive tumors appears to be much less frequent, 33% to 36%, in patients who had developed metastatic disease [14]. Consequently, Wapnir et al. suggests that NIS expression in metastatic breast tumors may have been altered by disease progression or concurrent therapies.

Due to the predominantly intracellular NIS staining reported in the literature, the prevalent view believes that differential NIS cell surface levels are mainly contributed by defective NIS cell surface trafficking [10,12-15] rather than differential NIS expression. However, as shown in Fig. 1, evident cell surface NIS staining with diffuse cytoplasmic NIS staining was found in the majority of tumors examined in our current study using #442 human antibody. In fact, only 10% (n=19) of the tumors had predominant intracellular NIS staining. It is clinically important to determine the mechanisms underlying variable cell surface NIS levels among

breast tumors such that appropriate strategies can be devised to increase cell surface NIS levels for radionuclide imaging and therapy.

Cross-reactivity could contribute to intracellular NIS immunostaining in breast cancer

Considering the inherent limitations of immunohistochemical staining, we conducted experiments to investigate the relevance of antibody cross-reactivity on intracellular NIS staining by examining the same tissue samples with multiple NIS antibodies. In this study, tissue sections from a Graves' disease thyroid case and two breast cancer cases were immunostained with #442 polyclonal, #836 polyclonal and VJ1 monoclonal NIS antibodies.

As shown in Fig. 2, NIS protein was predominantly detected at the cell surface in Graves' disease thyroid tissue using #442 affinity purified polyclonal antibody (Fig. 2A), #836 non-purified polyclonal antibody (Fig. 2B), as well as VJ1 monoclonal antibody that recognizes the extracellular domain of NIS (Fig. 2C). Both #442 and VJ1 antibodies detected minimal intracellular NIS staining in Graves' disease thyroid tissue, suggesting effective NIS cell surface trafficking in this tissue and/or little cross-reactivity. In comparison, non-specific diffuse cytoplasmic staining was more apparent with the #836 non-affinity purified antibody.

In the invasive breast carcinomas (Fig. 2D and 2G), #442 antibody also recognized cell surface NIS with the presence of diffuse cytoplasmic staining. However, #836 NIS antibody barely detected any cell surface NIS, yet detected predominantly cytoplasmic staining in the same breast tumors (Fig. 2E, 2H), despite its ability to detect prominent cell surface NIS in the Graves' disease thyroid tissue positive control (Fig. 2B). Therefore, the incidence of predominant intracellular staining of NIS in breast cancers may be overestimated. The discrepancy of NIS staining patterns between breast tumors and Graves' disease thyroid tissue is best explained by differences in signal-to-noise ratio. A better signal-to-noise ratio observed in Graves' disease thyroid tissue appears to be contributed by the combination of more abundant cell surface NIS and less non-NIS proteins that cross-react with #836 antibody tumors than in breast tumors. Surprisingly, VJ1 antibody fails to recognize NIS in the same breast tumors (Fig. 1F, 1I), indicating that the VJ1 epitope is either not accessible due to post-translational modifications or has a different tertiary structure in breast tumors.

Ideally, the identity of target proteins should be confirmed by multiple antibodies against non-overlapping epitopes. Nearly all six immunohistochemical studies reported in Table 1, including our own, relied on a single human NIS antibody to examine NIS expression in breast tumors. One study utilized more than one NIS antibody for immunostaining, yet the consistency of staining patterns among them was not discussed [10]. To date, almost all custom-made and commercial human NIS antibodies have been generated against the intracellular C-terminus of human NIS, except the VJ1 monoclonal NIS antibodies that recognize the extracellular domain of human NIS. Unfortunately, NIS in breast cancer was not recognized by the VJ1 antibody in this study. It is also important to note that the quality of polyclonal antibodies against the same antigen is not always consistent. In the absence of additional antibodies that recognize other epitope regions of NIS in breast tumors, it is important to confirm the identity of intracellular NIS staining and exclude the possibility of antibody cross-reactivity by other means, such as Western blot analysis of deglycosylated samples (see below).

NIS identity could be confirmed by Western blot analysis with deglycosylation

Western blot analysis is useful for identifying proteins of interest with expected molecular weights and is more quantitative than immunohistochemical staining. However, NIS is a glycoprotein and has varied degrees of glycosylation resulting in different molecular weights among tissues. Performing Western blot analysis with samples deglycosylated by PNGase-F, an enzyme that cleaves N-linked carbohydrates, not only allows us to verify that the detected

protein is a glycoprotein, but it also verifies the ~60 kDa molecular weight of non-glycosylated human NIS protein.

We conducted a pilot study to screen for the presence of NIS protein in a panel of human breast epithelial cell lines by Western blot analysis using three NIS antibodies. As shown in Fig. 3, a protein of 110 kDa was detected by the affinity purified #331 human NIS antibody (Fig. 3A) as well as the PA716 rat NIS antibody (Fig. 3C) in MCF-10A pre-malignant breast cells. However, the detected protein could not be deglycosylated upon PNGase-F treatment suggesting that the recognized protein is not a glycoprotein. The absence of NIS-mediated RAIU in MCF-10A cells (Fig. 4) further supports that the detected 110 kDa protein is not NIS glycoprotein. In comparison, NIS glycoprotein of 90 kDa detected in Graves' disease thyroid tissue with #331 (Fig. 3A) and FP-13 (Fig. 3B) human NIS antibodies was converted to 55 kDa after deglycosylation. Similarly, NIS protein detected in retinoic acid-treated MCF-7 human breast cancer cells [21] was converted to ~60 kDa after deglycosylation (data not shown).

To date, only one study has performed Western blot analysis to detect NIS protein in breast cancer in which a 97-kDa band was detected in breast tumors as well as Graves' disease thyroid tissue [12]. However, NIS identity was not confirmed by deglycosylation in this study by Upadhyay et al. [12]. Taken together, our study warrants that NIS identity should be further confirmed by Western blot analysis of deglycosylated proteins.

Conclusions

While NIS is detected in the majority of breast cancers, only a small fraction of NIS-positive breast cancers have detectable radionuclide uptake activity. We believe that the discrepancy is mainly contributed by variable cell surface NIS levels. To screen for patients most likely to benefit from radionuclide imaging, we must determine the level of cell surface NIS and the percentage of NIS-expressing tumor cells required for detection of radionuclide uptake *in vivo*.

For patients with tumors that have insufficient cell surface NIS levels, appropriate strategies should be devised to selectively increase cell surface NIS levels for effective radionuclide imaging and therapy. To accomplish this objective, it is essential to uncover whether differential cell surface NIS levels among breast tumors are contributed by either differential NIS expression or by cell surface trafficking defects. Consequently, NIS identity of intracellular staining should be confirmed by developing additional NIS antibodies against non-overlapping epitopes. Alternatively, conducting a parallel experiment with Western blot analysis of tumor samples in the presence and absence of PNGase-F will increase our confidence in the identity of intracellular NIS staining. If NIS cell surface trafficking defects are commonly found in breast tumors, selectively increasing NIS expression in breast tissues may not be sufficient to facilitate NIS-mediated radionuclide imaging and therapy. Instead, research efforts should focus on facilitating NIS cell surface trafficking.

In summary, the current literature inspires the possibility of NIS-mediated radionuclide imaging and targeted therapy for patients with breast cancer. However, no attempt has been made to correlate cell surface NIS protein levels with NIS-mediated radionuclide uptake activity *in vivo*, which is pertinent to define the cell surface NIS levels required for radionuclide imaging as well as to recognize possible defects impairing NIS activity. Taken together, this study warrants the importance of quantitative approaches for future research such that the application of NIS-mediated radionuclide imaging and targeted therapy can be realized for breast cancer patients.

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Figure 1.

Variable cell surface NIS protein levels among breast cancers. Breast tumors were immunostained with #442 polyclonal human NIS antibody and scored according to the level of cell surface NIS expression. Representative images of breast tumors scored as 0 (28%, n=54), 1+ (43%, n=83), 2+ (21%, n=40) and 3+ (8%, n=15) are shown.

Figure 2.

Inconsistent NIS staining in breast tumors by various human NIS antibodies despite consistent NIS staining in Graves' disease thyroid tissues. Graves' disease thyroid tissue (A-C) and two representative invasive breast carcinomas (D-F and G-I) were stained with #442 (A, D, G), #836 (B, E, H) and VJ1 (C, F, I) human NIS antibodies. (A, D, G) Strong plasma membrane staining was evident in Graves' disease thyroid tissue as well as representative malignant breast tumors with the affinity purified #442 polyclonal human NIS antibody. Arrows denote plasma membrane staining. (B, E, H) #836 polyclonal human NIS antibody resulted in diffuse non-specific staining in breast tumors, whereas Graves' disease thyroid tissue had intense basolateral membrane staining with some cytoplasmic staining. (C, F, I) Breast tumors showed negative immunostaining with VJ1 monoclonal antibody in contrast to the intense plasma membrane staining observed in Graves' disease thyroid tissue. (400x magnification).

Figure 3.

Deglycosylation is instrumental to confirm the identity of NIS glycoprotein by Western blot analysis. Western blot analysis using (A) #331 polyclonal human NIS antibody, (B) FP-13 monoclonal human NIS antibody and (C) PA716 polyclonal rat NIS antibody was performed in a panel of human breast cell lines (MCF-10A: a pre-malignant human breast cell line, MB-231, SK-BR-3, and MCF-7: human breast cancer cell lines) along with Graves' disease thyroid tissue (Grv Thy) as a positive control. NIS in Graves' disease thyroid tissue was detected by both (A) #331 human NIS and (B) FP-13 human NIS antibodies, but not by (C) PA716 rat NIS antibody, indicating that the PA716 rat NIS antibody does not recognize human NIS. The identity of NIS glycoprotein in Graves' disease thyroid was further confirmed by the

detection of deglycosylated NIS upon PNGase-F treatment. In comparison, the molecules detected by (A) #331 human NIS or (C) PA716 rat NIS antibodies in MCF-10A cells were unknown as they were not glycoproteins.

Figure 4.

The identity of functional NIS is best demonstrated by radioiodide uptake assay. The lack of NIS-mediated RAIU activity in breast cancer cells is consistent with the finding that NIS protein is absent in MCF-10A, MB-231, SK-BR-3 or MCF-7 cell lines. FRTL-5 rat thyroid cells were used as a positive control for RAIU.

Table 1
Summary of immunohistochemical studies for NIS detection in breast cancer

Reference	% NIS Positive Tumors	Antibody	Control	Negative	Inclusion of Confirmatory Methods
10 [#]	83% (DCIS, n=6) 87% (IC, n=23)	Polyclonal Ct-1 (aa 618-633) Polyclonal Ct-2 (aa 631-643) Monoclonal (aa 598-621)	Salivary gland	Peptide inhibition IgG control	N
11 ^{NS}	90% (42% strongly positive) (IC, n=50)	Monoclonal NIS BrA 10-11 (Epitope NS)	Thyroid papillary carcinoma	Normal breast	N
12 [#]	87% (IC, n=12)	Monoclonal NIS FP5a (aa 625-643)	Graves' thyroid	2° antibody only	Western blot (n=5)
13 [#] *	88% (53% strongly positive) (DCIS, n=17) 76% (40% strongly positive) (IC, n=91) 68% (34% strongly positive) (DCIS, n=41) 66% (29% strongly positive) (IC, n=137)	Polyclonal (aa 631-643)	Salivary gland	Peptide inhibition 2° antibody only	N
14 [#]	36% (index tumor, n=14) 33% (metastatic, n=9)	Polyclonal (aa 631-643)	Salivary gland	Peptide inhibition 2° antibody only	Scintigraphy
15 [*]	80% (NS, n=33)	Polyclonal 331 (aa 468-643)	Graves' thyroid	NS	N

NS, Not stated; DCIS, ductal carcinoma *in situ*; IC, invasive carcinoma

[#]Conventional tissue sections

* TMA breast tumor cores