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ORIGINAL ARTICLE

Expression of sodium iodide symporter in human breast tissues

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Summary

Purpose: *Sodium iodide symporter (NIS) expression in breast cancer (BC) tissues suggests the possibility of using radioiodine for diagnostic and therapeutic purposes. This study evaluated NIS protein expression in primary BC samples and its association with BC prognostic markers and thyroid functional parameters.*

Methods: *Fifty-six breast tissue samples from 52 operated women (41 BC, 11 benign breast diseases (BBD) and 4 peritumoral adjacent to the carcinoma tissues samples) were analyzed by immunohistochemistry using monoclonal anti-NIS antibody. Measurements of baseline levels of thyroid hormones and antibodies were also analysed in association with NIS protein expression.*

Results: *NIS presented positive cytoplasmic immunoreactivity in the majority of BBD samples (45%) and in 3 out of 4 breast tissues adjacent to carcinoma. Immunoreactivity was extremely faint in BC samples. Mean thyroid hormones levels and thyroid antibodies positivity did not differ statistically between patients with NIS positive (faint expression) and NIS negative BC. NIS expression appeared to be independent of estrogen (ER) and progesterone receptor (PR) expression as well as of lymph node status.*

Conclusions: *BC samples expressed only faintly and mainly cytoplasmic NIS protein, suggesting the presence of a non-functional NIS protein. The hypothesis of the down-regulation of NIS expression during carcinogenesis cannot be excluded.*

Key words: *breast cancer, sodium iodide symporter, thyroid, thyroid antibodies, thyroid hormones*

Introduction

NIS-sodium iodide symporter (also called SLC5A5)- is a transmembrane glycoprotein that resides in the basolateral membrane of thyroid epithelial cells organized into 13 membrane-spanning domains with three N-linked carbohydrates [1,2]. In normal thyroid tissue, NIS mediates active iodide transport into cells. Thyrotropin (TSH) increases the expression of genes involved in thyroid iodide metabolism and thyroid hormone synthesis including NIS, thyroglobulin (Tg) and thyroperoxidase (TPO) [3]. TSH stimulates not only *NIS* gene expression, but also the translocation of NIS to the plasma membrane of normal thyroid epithelial cells. Negative feedback regulation is mediated through inhibition of the TSH secretion by the accumulation of thyroid hormones at pituitary and hypothalamic level [4,5]. *In vitro* and *in vivo* experiments have demonstrated that Tg is a potent suppressor of NIS mRNA expression [6]. Data show that in thyroid carcinomas, NIS mRNA levels were lower compared to normal thyroid cell and located mainly in the cytoplasm, thus lacking the ability to transport and absorb iodine in patients with thyroid carcinomas [7].

Various extra-thyroidal tissues express NIS mRNA and/or protein including salivary gland,

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gastric mucosa, mammary gland, ciliary body of the eye and the choroid plexus [4,8]. However, plasma membrane NIS immunopositivity was confirmed only in salivary ducts, gastric mucosa and lactating mammary cells [9]. It has been established that NIS expression in the mammary gland is induced during lactation [10,11]. However, there are distinct mechanisms regulating the iodide uptake in the thyroid and mammary glands. For instance, fetoplacental estrogens as well as oxytocin and prolactin are potent stimulators of NIS expression in the mammary gland, but not in the thyroid gland [10,11].

Additionally, iodide uptake has been also detected in non-lactating mammary glands. It has been reported that the NIS gene is expressed in BC, suggesting the possibility of radionuclide imaging and therapy [11,12]. In triple-negative BC tissues, defined by the absence of ER, PR and human epidermal growth factor 2 (HER2) receptors expression [13], NIS was strongly expressed in plasma membrane in 65%, suggesting a potential role of NIS-directed ^{131}I-radioablative strategies in these patients [14]. Recently, it has been reported that the radioiodine uptake in BC samples was significantly higher as compared to that observed in the normal tissue from the same patients [15].

However, the regulatory mechanisms of *NIS* gene expression in BC and its exact role are still not well understood. The primary aim of this study was to evaluate the NIS expression in BC by immunochemical analysis, and secondarily, its correlation with established prognostic factors in BC.

Methods

Written informed consents were obtained from each participant prior to their inclusion in the study. The study protocol was approved by the Scientific and Ethics Committee of Laiko Hospital of the University of Athens according to the Helsinki declaration.

Study cohort

Fifty-six formalin-fixed, paraffin-embedded breast tissue samples were selected from the archives of the 1st Pathology Department of the University of Athens. All the breast samples were obtained from 52 operated women hospitalized in the 2nd Surgical Department of Laiko Hospital (University of Athens), between January 2010 and March 2014 with a mean age 49±6.6 years (median 50). Samples were selected according to the histological reports based on Nottingham (modified by Bloom-Richardson) and WHO criteria [16] and consisted of 41 BCs (31 invasive ductal carcinomas, 6 ductal carcinomas *in situ* /DCIS, and 4 lobular infiltrative carcinomas), 11 BBD (4 papillomas and 7 fibroadenomas) and 4 samples adjacent to carcinoma.

From the 41 BC samples, 6 (14.6%) were classified as grade I, 19 (46.3%) as grade II and 15 (36.6%) as grade III. The 65.8% (27 out of 41) of BC samples were positive for ER and PR and from the remaining 14 BC samples 9 were triple-negative (ER-, PR- and HER2-).

Antibodies for immunohistochemistry

For the immunohistochemical analysis of the NIS protein, we used the same mouse monoclonal anti-NIS antibody (clone FP5A) which had been already used by other authors [18] for the NIS detection by immunohistochemistry. This antibody identifies a peptide corresponding to the amino acids 625-643 of the NIS protein (purchased from Thermoscientific, Rockford, USA) [17,18].

ER and PR expression was immunohistochemically detected in breast tissues with the commercially available ER antibodies (clone EP1) and PR antibodies (clone PgR636) (DAKO, Glostrup, Denmark) according to ASCO recommendations [19]. HER2 expression was detected immunohistochemically using the anti-HER2 monoclonal antibody (CB11, DAKO) according to ASCO recommendations [20].

Immunohistochemistry for NIS detection

Breast tissues were fixed in 10% neutral formalin and embedded in paraffin. Sections (3 μm thick) of these tissues were put on slides. Sections were deparaffinized, rehydrated and immunostained using an automated immunostainer (PT link of DAKO) at standardized duration and temperature (PT T buffer : 100% citrate, pH=9.0, T= 92 °C for 1 h). The slides with the breast tissues were incubated in 3% peroxide for 15 min to quench endogenous peroxidase. After pre-treatment, slides were incubated for 30 min with human anti-NIS Ab at 1/200 dilution. Immunostaining of all the samples was performed with a basic DAB Detection kit (Thermoscientific, Rockford, USA). After washing with buffer, all slides were counterstained with haematoxylin (Mayer buffer haematoxylin). Immunohistochemical assessment of NIS was performed in the lesion and in some cases (4 samples) in the adjacent normal breast tissues. Normal thyroid gland tissue sections were used as positive controls.

Grading of the staining

The score of the grading of the immunostaining was based on the intensity of the labeling and the subcellular localization of the staining (membranic or intracellular) as previously described by other authors [9]. The intensity of the expression of NIS protein was evaluated as a variable score (0=negative, 1=low, 2=moderate, and 3=intense expression). Immunoreactivity was characterized as either weak or strong but

Table 1. NIS expression of the studied samples (Fisher's exact test: p=0.426 for NIS expression between BC and BBD)

Breast pathologies	*Total number of samples*	*Pos NIS expression, N (%)*	*Neg NIS expression N (%)*	*Intensity of NIS expression*		
				1+	*2+*	*3+*
BC samples	41	13/41 (32)	18/41(68)	1	12	-
Ductal infiltrative cancer	31	8 (25.8)	23 (74)	1	7	-
Lobular infiltrative cancer	4	1 (25)	3 (75)	-	1	-
DCIS	6	4 (66.6)	2 (33.3)	-	4	-
BBD samples	11	5/11 (45)	6/11(55)	-	4	-
Papillomas	4	3 (75)	1 (25)	-	3	-
Fibroadenomas	7	2 (28.5)	5 (71)	-	2	-
Peritumoral tissue	4	3 (75)	1 (25)	-	2	1

NIS: sodium iodide symporter, BC: breast cancer, BBC: breast benign disease, DCIS: ductal carcinoma in situ

had to encompass at least 20% of cells [9]. When plasma membrane immunoreactivity was noted, it was scored as strong if >10% of cells demonstrated this feature either alone or in the presence of intracellular immunoreactivity.

Laboratory measurements

Baseline thyroid functions tests were also performed in the studied population during their hospitalisation before any surgery or other treatment for BC. TSH (normal range: 0.3-4.0 mU/l) was measured with the double enzyme radioimmunoassay (RIA) 'sandwich' method (TSH-DiaSorin –CTK- 3 Kit, Italy). Free thyroxine (fT4) (normal range: 10-25 pmol/l) and free triiodothyronine (fT3) (normal range: 2.3-5.3 pg/ml) were measured in the serum with the double enzyme radioimmunoassay 'sandwich' RIA method (ThermoScientific kit, USA). Auto-antibodies against Tg (positivity > 10 mU/l) and TPO (positivity > 50 mU/L) were also measured with the RIA method (ThermoScientific kit, USA). All the measurements of TSH, fT3, fT4, TPO Ab and Tg Ab were performed at 'Laiko' Hospital, Athens, Greece. The TSH receptors antibodies (TRAK) (positivity > 2 IU/l) were measured with the receptor radioassay (RRA) method (TRAK human of Thermofisher-BRAHMS 101.5 kit, Germany) in the laboratory of Endocrinology of the Evgenidion Hospital, Athens, Greece.

Statistics

Continuous variables (thyroid function tests) were evaluated initially by the Kolmogorov-Smirnov test. Due to lack of normal distribution, variables (values of fT3, fT4, TSH) were evaluated by the Kruskall-Wallis test. Continuous variables (values of TSH, fT3, fT4) were summarized numerically by means ± SD. Categorical parameters (positivity of thyroid antibodies, grade, stage and hormonosensitivity of breast cancer) were evaluated by the Fisher's exact test because of small sample (cells with less of 5 frequencies). Results were considered significant at $p<0.05$. Statistical analyses were performed with SPSS software, version 20.

Results

NIS expression in breast tissues samples

Normal thyroid cells, used as control, presented normal intensity (+3) cell membrane NIS positive immunoreactivity (Figure 1a). In contrast, NIS immunoreactivity was exclusively cytoplasmic in epithelial cells of all the studied BBD (Figure 1b), BC (Figure 1c), and breast tissue adjacent to BC samples (Figure 1d). NIS presented positive immunoreactivity in 5 out of 11 BBD samples and in the 3 out of 4 breast tissue samples adjacent to BC samples. NIS immunoreactivity was faint in 13 out of 41 BC samples and negative in the remainder 28 BC samples. Statistical analysis revealed no significant difference in NIS immunoreactivity between BC and BBD samples (p=0.426) (Table 1).

Association of NIS expression with thyroid function

TSH and fT4 mean levels were slightly higher in NIS-positive BC samples (2.9±1.65 for TSH and

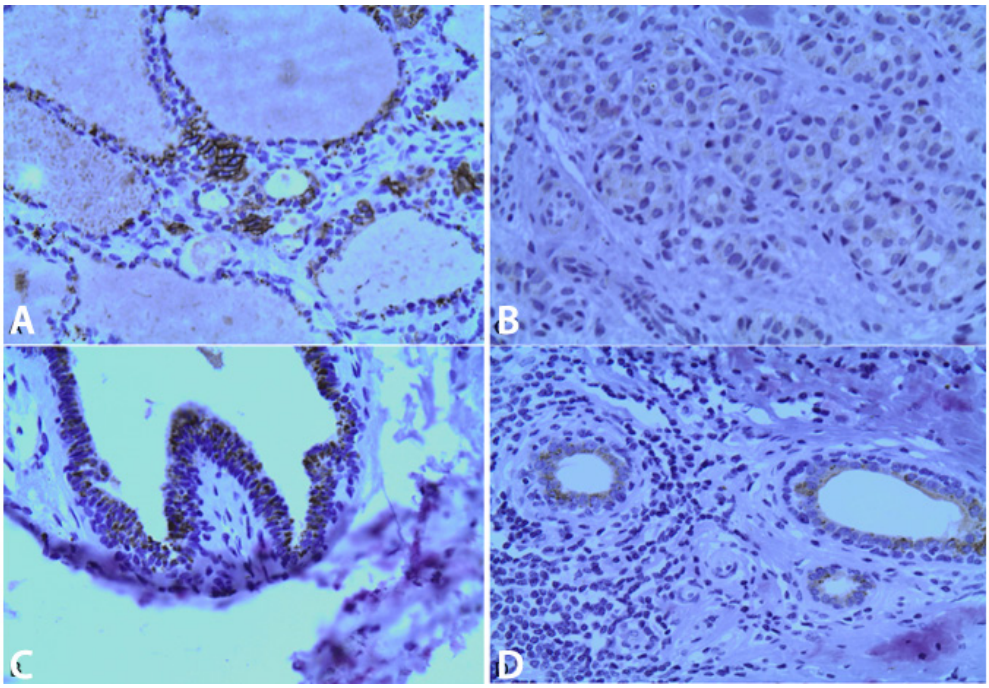

Figure 1. Immunohistochemical detection of NIS protein in 3 μm sections of breast and thyroid tissues. **A:** Normal thyroid tissue (positive control x 400) with membrane staining (NIS score: +3). **B:** Fibroadenoma (x 400) with cytoplasmic staining (NIS score: +2). **C:** Invasive breast carcinoma (x 400) with cytoplasmic staining (NIS score: +1). **D:** Normal breast tissue adjacent to carcinoma (x 400) with cytoplasmic detection of NIS staining (NIS score: +2).

17.10±2.37 for fT4) compared with NIS-negative BC samples (2.25±1.85 for TSH and 16.9±2.95 for fT4). Mean baseline levels of fT3 were similar in NIS-positive and NIS-negative BC (Table 2). Mean baseline thyroid hormones levels, as well as thyroid antibodies positivity, showed no significant differences between BC patients with positive and those with negative NIS immunostaining (Table 2). No statistical analysis was performed for BBD samples due to their small number .

Association of NIS expression with prognostic factors of BC

NIS immunoreactivity was indepedent of lymph node metastases status amd ER/PR expression in BC samples. More specificaly, 40% of the BC patients with lymph node metastases presented positive NIS immunoreactivity, compared

Table 2. Association of thyroid function tests (from patients with breast cancer) with NIS expression in the breast cancer samples

Thyroid function test/NIS expression	*NIS +*	*NIS-*	*p value*
TSH			
mean values±SD (BC)	2.9±1.65 (N=14)	2.25±1.85 (N=30)	p=0.588*
fT4			
mean values±SD (BC)	17.10±2.73 (N=10)	16.9±2.95 (N=26)	p=0.831*
fT3			
mean values±SD (BC)	3.12±0.743 (N=12)	3.22±0.762 (N=27)	p=0.726*
TPOAbs			
positive	3/7	4/7	p=0.281**
negative	3/14	11/14	
Tg Abs			
positive	1/6	5/6	p=1.0*
negative	1/10	9/10	

NIS: sodium iodide symporter, TSH: thyreotropin, fT4: free thyroxin, fT3: free triidothyronin, TPO Abs: thyroperoxidase antibodies, Tg Abs: thyroglobulin antibodies, BC: breast cancer, SD: standard deviation

* Kruskall Wallis, ** Fisher exact test

to 22% of the BC patients without lymph node metastases (p=0.285) (Table 3). Prevalence of NIS immunoreactivity was also similar in ER and PR positive BC samples (30%), compared with ER and PR negative BC samples (26%) (p=1.00). Of note, only 2 out of 9 triple-negative BC showed NIS expression.

Discussion

In the present study we found a positive immunoreactive cytoplasmic expression of NIS protein in BBD tissue samples and only a faint immunoreactivity in BC tissue samples. Among BC samples, NIS expression was independent of lymph node metastases and T stage and also of ER/PR expression. Although the function and regulation of NIS protein has been well established in thyroid tissue, iodide accumulation (I-) in extrathyroidal tissues, including salivary glands, gastric mucosa and lactating mammary gland has not been clearly studied [9]. Recent data showed that NIS is also expressed in several malignant tissues including ovary, lung, colon and endometrium [9].

Our data demonstrated only a very faint intracellular NIS protein expression in 32% of BC samples and negative immunoreactivity in the majority of BC samples. This contrasts other studies that have shown a strong immunohistochemical NIS protein expression in BC samples up to 88% in DCIS and 65-90% in invasive ductal BC using either polyclonal [9,14,21] or monoclonal anti-NIS antibodies [6]. In some cases, this expression was also confirmed with other techniques such as western blot analysis [21] and qRT-PCR [6]. In other cases, NIS was also strongly expressed in a significant proportion of triple-negative BC with a relatively increased I^{131}uptake [22]. In these studies, NIS staining in BC samples was found either in both plasma membrane and the cytoplasm [6,21] or only in the cytoplasm [9]. NIS expression in BC tissues led to the investigation of its expression in BC metastatic tissues [12]. Wapnir et al. [12] showed that 8 out of 23 patients with metastatic BC presented protein immunohistochemical NIS expression (with the use of polyclonal antibody) and 2 of them also exhibited iodide uptake. Similarly, tissues samples from brain metastases in BC patients showed cytoplasmic immunohistochemical expression of NIS protein in 75% and membranic expression in 23.8% [22].

In a recent study [18], the immunohistochemical analysis of NIS expression in BC samples using the same monoclonal antibody as in our study (monoclonal mouse anti-NIS, FP5A) also demonstrated a very low expression of NIS protein in BC

Table 3. Association of prognostic factors (tumor size, nodal metastasis and grade) in breast cancer samples with NIS expression

Prognostic factors/NIS expression	*NIS+ N (%)*	*NIS- N (%)*	*p value**
T stage			
T1	4/19 (21)	15/19 (79%)	0.274
T2	3/13 (23)	10/13 (77%)	
T3	4/7 (57)	3/7 (43%)	
T4	1/2 (50)	1/2 (50)	
N stage			
N+	6/15 (40)	9/15 (60)	0.285
N-	5/23 (22)	18/23 (78)	
ER/PR positivity			
-ER+/PR+	7/27 (26)	20/27 (74)	1.00
-ER-/PR-	3/10 (30)	7/10 (70)	
Grade			
I	2/6 (33.3)	4/6 (66.6)	1.00
II	5/19 (26)	14/19 (74)	
III	4/15 (26.6)	11/15 (73.3)	

FNIS : sodium iodide symporter, ER: estrogen receptors, PR: progesterone receptors
*Fisher's exact test

samples. In the same study, western blot analysis found that the fully glycosylated NIS protein was undetectable in BC cells; however, partially glycosylated NIS protein and a NIS fragment were present, corresponding to the samples with faint intracellular staining.

Obviously, there are conflicting results in the literature regarding the expression of NIS protein in BC. These differences among studies could be explained by the use of different and preferentially polyclonal antibodies in the majority of them. Therefore, the high percentages of NIS protein expression in BC could be related to a non-specific binding [9,21]. In addition, the detection of the NIS protein in the cytoplasm instead of plasma membrane, shows the lack of its functional capacity for I-transport into cells. This issue has already been addressed in studies with imaging confirmation, where iodide uptake activity in BC was insufficient in clinical practice as it was found in I^{123} SPECT images [9,11,12,23,24].

Concerning the NIS higher expression in BBD samples such as fibroadenomas, these data agree with previous studies showing increased NIS mRNA and/or protein levels with strong plasma membrane staining in fibroadenomas compared with malignant breast cells [6,25]. In some cases, higher NIS expression in fibroadenomas was also associated with an increased functional capacity with increased iodide accumulation, as it had been shown by imaging studies with whole body gamma camera scan I^{131}[26]. Yet, there are other studies with contradictory results, reporting lower NIS protein expression in normal and benign breast tissues samples compared with BC, suggesting that NIS might be upregulated during the malignant transformation of breast epithelial cells [9].

In any case, enhancing NIS expression in BC remains a challenging and interesting approach that could help in the studying of its functionality and utility for diagnostic and therapeutic purposes [27]. A number of potential regulators of NIS in BC has been identified. Among them, the unliganded estrogen receptor alpha (ERa) has been shown to activate mammary NIS transcription in ER positive breast cancer cells lines [28]. This is supported by the presence of an estrogen responsive element in the NIS gene promoter. A positive correlation has already been found between NIS protein expression and Retinoic Acid Receptor alpha (RARa) and ERa expression in BC [6], whereas in another study no relationship was found between NIS mRNA levels and hormonal receptors expression [29]. In our study, although the number of samples was small, NIS expression was found independent of ER/PR expression.

In vitro studies have also shown significant stimulation of NIS expression in cell lines exposed to thyroid hormones. In our data, NIS expression in BC tissues was not correlated with the plasma levels of thyroid hormones. In a recent study, NIS expression was induced by the combined exposure of all trans retinoic acid (ATRA) and T4, showing a synergistic increase greater than that observed with each individual factor alone [30]. This positive effect of T4 on mammary NIS expression observed in vitro contrasts the inhibitory role of T4 in thyroid NIS regulation [30].

The role of NIS as a potential prognostic marker in malignancies has already been shown in ovarian cancer, predicting a poor prognosis in these patients [5]. However in BC, the prognostic role of NIS is not well demonstrated. Our data are in agreement with previous studies that have shown that NIS expression was not correlated neither to BC grade [21] nor the ER/PR expression [21,29].

Conclusions

Immunohistochemical analysis showed that NIS protein expression was negative in the majority of the BC samples and only faint with exclusively cytoplasmic localization in 32% of them. The association of NIS expression with the prognostic markers of BC is not clear and no firm conclusions can be made based on a small sample in our study. Furthermore, we found that BBD tissues show a more frequent and intense cytoplasmic staining of NIS protein compared to BC tissues. Future studies should be focused on the enhancement of NIS expression and its trafficking from the cytoplasm to plasma membrane for a better understanding of its functional role in BC.

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