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Differential Expression of the Na^+/I^- Symporter by Endocrine Receptor Status: A Potential New Therapeutic Target in Estrogen Receptor Negative High Grade Breast Cancers

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Abstract

Purpose: Expression of the sodium-iodide (Na^+/I^-) symporter (NIS) in thyroid cancer accounts for the success of radioactive iodine (^{131}I) ablation of remnants or metastases following thyroidectomy. Breast cancer (BC) is one of the other malignancies that express functional NIS. The aim of the present study was to evaluate the expression and localisation of NIS according to BC subtype.

Materials and methods: Ninety predominantly high grade primary BC were independently evaluated for NIS expression and cellular localization (membranous and/or cytoplasmic) by immuno-histochemistry. mRNA expression of the NIS (SLC5A5) gene was analyzed across 18 publicly available whole genome microarray datasets including 3,091 primary breast tumors. NIS expression was compared with clinical and pathological tumor characteristics.

Results: Cytoplasmic (cNIS) and membrane (mNIS) immunoreactivity was more frequently observed in estrogen receptor (ER) and progesterone receptor (PR) negative (ER-/PR-) tumors (45% mNIS and 67% cNIS) than in ER and PR positive (ER+PR+) tumors (7% mNIS and 20% cNIS) ($p<0.001$). cNIS staining was not present in only 4% (1/25) of the tumors with mNIS staining.

NIS mRNA expression was higher in ER negative tumors defined by local pathological ER evaluation ($p=0.013$) or microarray classification ($p=0.011$).

HER2 status had no influence on NIS expression regardless of the analytical method considered.

Conclusions: NIS expression is increased in ER negative breast cancer. ER negative breast cancer should be targeted for clinical studies evaluating radioactive iodine therapy including strategies to mobilize cytoplasmic NIS to the cell membrane and enhance the activity of ^{131}I in these cancers.

Keywords: Breast cancer; Natrium-iodide symporter; Endocrine receptor

Introduction

The sodium/iodide (Na^+/I^-) symporter (NIS) is a transmembrane glycoprotein that transports iodide from the bloodstream against its concentration gradient into thyroid follicular cells for the biosynthesis of thyroid hormones triiodothyronine (T3) and thyroxine (T4). Functional NIS remains expressed in thyroid cancers and accounts for the success of radioactive iodine (^{131}I) in the (detection and) treatment of thyroid metastases after thyroidectomy.

NIS is also normally expressed in the breast during late pregnancy

and lactation [1,2] and breast cancers (BC) also express functional NIS. In animal models, primary breast cancers actively accumulate iodide by the NIS symporter *in vivo* [3,4] and up to 90% of invasive breast carcinomas have been reported to express cell surface (mNIS) and/or predominantly intracellular (cNIS) NIS protein [3,4].

The localisation of NIS staining may have important therapeutic implications as membrane NIS expression has been reported to suggest greater functional activity of the NIS than its expression in the cytoplasm alone. In a recent series of 23 triple negative (ER-PR-HER2-) primary breast tumors, 15 tumours (65.2%) expressed NIS, with 11 tumors (47.8%) exhibiting strong immunostaining but only four tumors were reported to have detectable membrane NIS [5].

Given the lack of therapeutic options for ER negative breast cancer, NIS-targeted radioactive iodine therapy warrants further evaluations in breast cancer. In order to select patients for clinical studies of radioactive iodine therapy, a better understanding of the biology of NIS in breast cancer is needed.

The aim of this study was to evaluate NIS expression (using immunostaining and gene techniques) and localisation (using immunostaining technique) across breast cancer subtypes defined by their ER, PR and HER2 expression.

Material and Methods

Tumour materials

In a first phase, NIS immunostaining in BC was developed in our institution in collaboration with B.F. (Hopital Ambroise Pare, France). An unselected series of pathological materials (histological blocks) from 23 patients of our institution was sent to her laboratory for NIS evaluation by immunohistochemistry. This initial series consisted of 3 patients with thyroid cancer and 20 with invasive BC including 7 women with ER-PR-HER2- BC, 11 ER+PR+HER2- and 2 ER-PR-HER2+. Her preliminary analysis was consistent with prior observations that NIS immunostaining was more frequently observed in ER-HER2- breast cancer.

In a second phase, the pathological materials of additional 70 patients with primary breast cancer who underwent surgical excision at our institution from 2003 to 2008 were selected for further analysis. Since nearly all ER-HER2- breast cancers are high grade, only histological grade III tumors (41 ER- and 29 ER+) were selected for further review. Pathological materials from patients who underwent pre-operative endocrine and/or chemotherapy treatments were excluded. Data from six men diagnosed with breast cancer (5 ER+ and 1 ER-) were also included.

The characteristics of the patients with breast cancer are shown in **Table 1** with stratification according to ER positivity. Except for immunostaining of the proliferation antigen Ki67, no differences in the biological and pathological characteristics were observed.

Immunohistochemical staining for NIS

Unstained slices were deparraffinized in xylene. Endogeneous peroxydase activity was blocked with 3% H_2O_2 solution for 10 minutes. Antigen retrieval was used with pH6 citrate buffer (10mM Sodium Citrate, 0.05% Tween 20) at 97°C for 40 minutes. The tumor slices were then incubated in a pH6 citrate buffer at room temperature (RT) for 20 minutes. The incubation with the primary antibody was performed at room temperature (hNIS 1/320). For the secondary antibody the kit LSAB from DAKO was used. DAB was used as chromogen. Counterstaining with Hemalun was performed. For positive controls, tissues from Graves' disease patients as well as from metastatic (nodal or distant) and/or primitive thyroid carcinomas scintigraphically proven to take up iodine-131 were used.

NIS antibodies

The PA795 polyclonal human NIS antibody raised against the human NIS peptide (amino acids 630-643) was kindly provided by Dr. Bernard Rousset, Institut National de la Santé et de la Recherche Médicale, Lyon, France [6].

Scoring of immunohistochemical NIS staining

Slices were analyzed by light microscopy by two experienced breast cancer pathologists (DL, NS) who were not aware of the ER and HER2 status of the primary tumour. The percentages of tumour cells with cytoplasmic (cNIS) and membranous (mNIS) were recorded. The following scoring system was used.

The level of cell membrane NIS (mNIS) in each case was scored on a scale of 0, 1+, 2+ and 3+ as follows:

- 0 if no cancer cells were stained
- 1+ if 1 to 3% of cells were stained
- 2+ if 4 to 9% of cells were stained
- 3+ if 10% or more of cells demonstrated this feature

Cytoplasmic staining (cNIS) was also scored on a scale of 0, 1+, 2+ and 3+ as follows:

- 0 if no cancerous cells were stained
- 1+ if 10% or less of cells were positive
- 2+ if between 10 and 30% of cells demonstrated this feature
- 3+ if 30% or more of cells demonstrated this feature

Gene Expression Data

Clinical and Gene expression data from 18 publicly available datasets including 3,091 primary breast cancer datasets were collected from public databases and/or authors' websites. Since the published reports included overlapping datasets, redundant patients were manually removed. Normalized microarray data (log 2 intensity in single-channel platforms or log 2 ratio in dual-channel platforms) were used as published by the original studies. Hybridization probes to the NIS gene (Entrez Gene ID=SLC5A5) were identified through sequence alignment against RefSeq mRNA in the (NM) subset, as in Shi et al. [7], using RefSeq and Entrez database version 2007.01.21. Breast cancer molecular subgroups were defined using a previously reported method [8,9]. The three main subgroups defined were: ER- HER2- , HER2+ over-expressing and ER+HER2- (luminal).

Table 1 Biological and pathological characteristics (N=90).

	ER + (N=41)		ER /-/ (N=49)		P-value
Age (in years)					0.55
mean ± std	57 ± 13		58 ± 13		
median (min-max)	57 (35-79)		59 (31-81)		
Tumor size (N=83)					0.90
≤ 2.0 cm	27	69%	31	70%	
2.1 – 5.0 cm	12	31%	12	27%	
>5.0 cm			1	2%	
unknown	2		5		
Her-2/neu*					0.78
Negative	33	80%	41	84%	
Positive	8	20%	8	16%	
Grade (N=87)					0.41
1	2	5%	-	-	
2	4	10%	4	9%	
3	34	85%	43	91%	
unknown	1		2		
KI67 (N=88)					<0.01
mean ± std	27 ± 15		46 ± 24		
median (min-max)	26 (5-60)		40 (5-90)		
≤25%	20	50%	11	23%	
>25%	20	50%	37	77%	
Not done	1		2		
Nodal status					0.25
Negative	26	63%	37	76%	
Positive	15	37%	12	24%	

*Her-2/neu is considered positive in case of amplification.

Statistical Methods

The χ2 test and Fisher exact tests were used to test compare categorical data. The Student t-test, Mann-Whitney, and the Kruskal-Wallis tests were used to compare continuous data. To assess the association between two ordinal variables, Stuart's tau-c and the Spearman correlation were calculated (range from -1 to 1). All reported p-values are two-tailed. Although multiple comparisons were performed, we considered a nominal *p*-value<0.05 as significant since all of the analyses were exploratory. Statistical analysis was performed using SAS version 9.2 (SAS Institute, Cary, North Carolina) and the SPSS statistical software package (SPSS Inc. Chicago, IL) version 15.0.

Results

Characteristics of mNIS and cNIS according to ER status

Figures 1a-1g demonstrates examples of positive and negative NIS staining by cellular location and compared to samples from thyroid cancer and Graves disease.

Irrespective of the number of cells stained, membrane (mNIS) and cytoplasmic (cNIS) NIS staining was more frequently observed in ER-PR-negative breast cancers (67% cNIS+ and 45% of mNIS+) than in ER+PR+ breast cancers (20% cNIS+ and 7% mNIS+) (**Table 2**) (*p*<0.01)

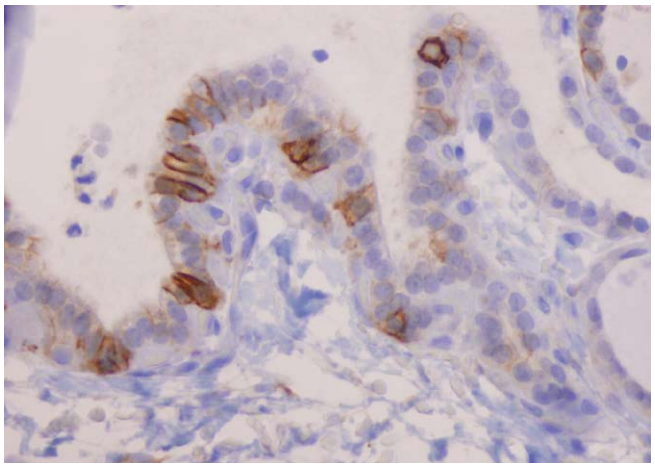

Figure 1a Positive control in thyroid Grave's disease.

If more stringent criteria are used to define the tumor samples as NIS positive (≥2+ or 3+ only), the difference between the ER-PR- and ER+PR+ groups remained statistically different (*p* value <0.01: (**Table 2)**.

In tumors with positive cNIS or mNIS staining (1+ to 3+), no

Table 2 Number of tumors and percentages of cells* with cytoplasmic (cNIS) or membrane (mNIS) Na+/I- symporter staining according to HR status (N=90).

	ER + PR+ (N=41)		ER /-/ PR /-/ (N=49)		(/-/) vs. (1+, 2+ or 3+) P-value	(/-/ or 1+) vs. (2+ or 3+) P-value	(/-/, 1+ o 2+) vs. 3+ P-value
	N		N				
cNIS							
/-/	33	80%	16	33%	<0.01	<0.01	0.02
1+	3	7%	8	16%			
2+	1	3%	11	22%			
3+	4	10%	14	29%			
Percentages of cells cNIS + (N=41)*							
mean ± std	30 ± 22		27 ± 19				
median (min-max)	30 (5-60)		20 (7-80)				
mNIS							
/-/	38	93%	27	55%	<0.01	<0.01	0.01
1+	1	2%	5	10%			
2+	-	-	6	12%			
3+	2	5%	11	22%			
Percentages of cells mNIS + (N=25)*							
mean ± std	7 ± 5		9 ± 8				
median (min-max)	10 (1-10)		9 (1-30)				

* among NIS positive tumors (1+, 2+ or 3+)

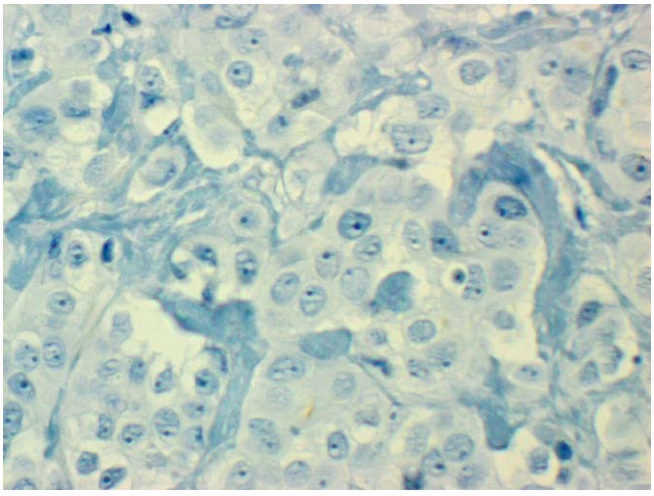

Figure 1b NIS negative breast cancer.

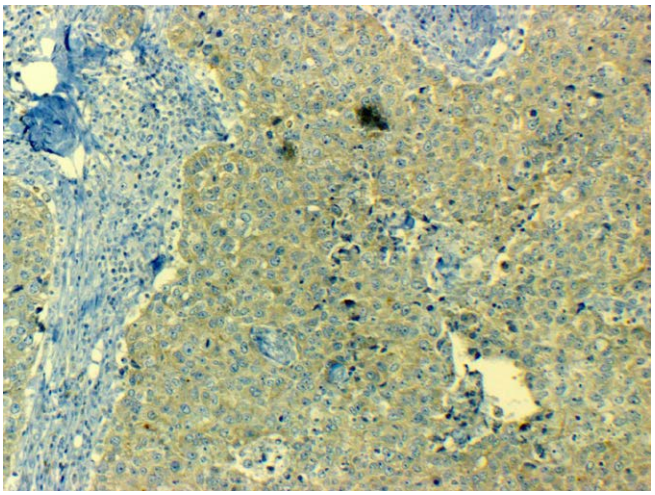

Figure 1c Positive cytoplasmic staining in more than 30% of the cells (cNIS 3+) without membrane staining (mNIS 0)

difference in the percentages of stained cells could be detected between ER-PR- and ER+PR+ cases {median 20 in ER-PR-cNIS+ tumors vs. median 30 in ER+PR+cNIS+ tumors (p=0.93); median 9 in ER-PR-mNIS+ tumors vs. median 10 in ER+PR+mNIS+ tumors (p=0.93)}.

In the 41 cNIS+ tumors, the median percentage of cells stained at the level of their cytoplasms was 20%, whereas in the 25 mNIS+ tumors median 10% of the cells were stained at the level of the membranes. However, when viewed as strongly positive, 9 (50%) of the 18 cNIS 3+ tumors had at least 50% of the cells stained (max. 80% stained) and 4 (30%) of the 13 mNIS 3+ tumors had at least 20% of the cells stained (max. 30% stained).

No statistically significant difference in the rate of cNIS+ or mNIS+ could be found between HER2 amplified an HER2 non amplied tumors: 44% (7/16) cNIS+HER2+ vs. 46% (34/74) cNIS+HER2- (p=1.0) and 25% (4/16) mNIS+ HER2+ vs. 28% (21/74) mNIS+HER2- (p=1.0).

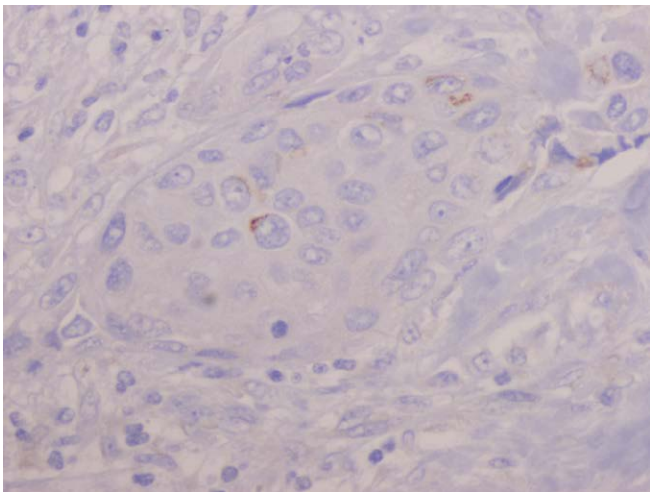

Figure 1d Case scored mNIS 0 but with less than 10 % of cytoplasmic staining (cNIS 1+).

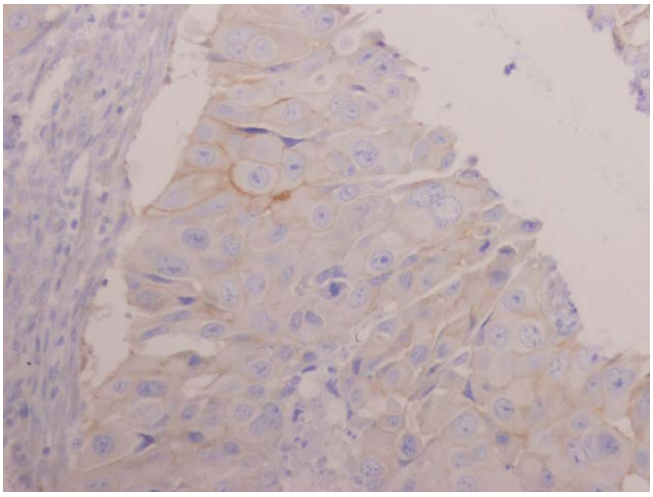

Figure 1e Case score cNIS 1+ but with 4 to 9% of the cells mNIS positive (mNIS 2+).

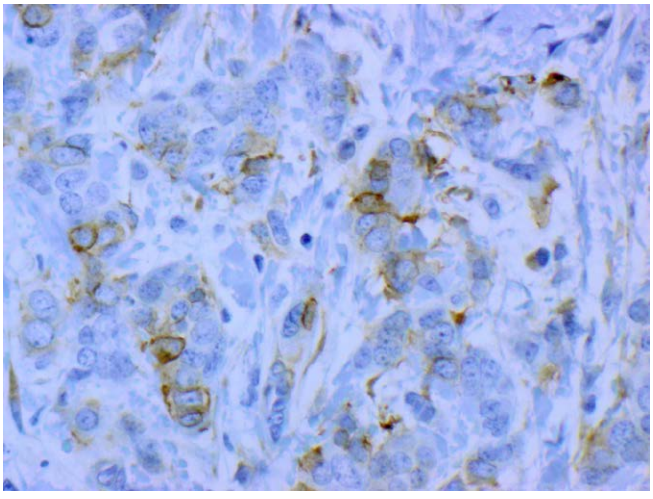

Figure 1f Case scored cNIS 1+ but with 1-3% of the cells mNIS positive (mNIS 1+).

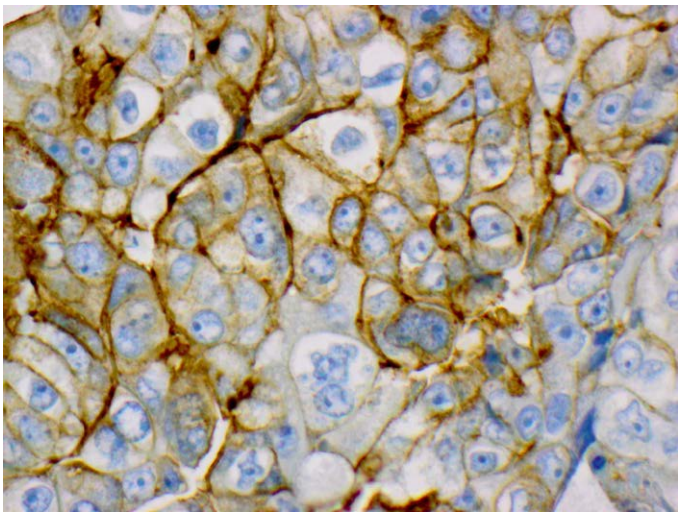

Figure 1g case scored cNIS 2+ (10 to 30% of cells positive) and with more than 10% of the cell mNIS positive (mNIS 3+).

Stratified by HER status, a higher rate of cNIS+ or mNIS+ was present in the ER-PR- cases, e.g. when restricting to the 16 HER2 positive tumors: 75% (6/8) cNIS+ in ER-PR- tumors compared to 13% (1/8) in ER+PR+ tumors (p=0.04) (**Table 3**).

With regard to our male patients, five had HR+ Neu- tumor : three were NIS negative and two were cNIS+ mNIS-. One man had HR- HER2 3+ tumor and was strongly cNIS (more than 50% of the cell stained) and mNIS (more than 20% of he cell stained) positive.

Correlation between cNIS and mNIS immunostaining (Tables 3 and 4)

cNIS+ was absent in only 1 out of 25 mNIS+ tumors (4%).

Strong mNIS staining (scored 3+) was observed in only a single case among the 49 with absent cNIS patients but in 3 (25%) of the 12 cNIS scored 2+ and 6 (33%) of the 18 cNIS scored 3+. The Spearman correlation between cNIS and mNIS was 0.64 (95% CI, 0.51-0.78); Stuart's Tau-c was 0.41 (95% CI, 0.29-0.52).

In ER negative patients, there was a clear relationship between the cNIS scoring and the mNIS positivity; 0/16 if cNIS 0, 4/8 (50%) if cNIS 1+, 7/11 (64%) if cNIS 2+ and 11/14 (76%) if cNIS 3+. Spearman correlation between cNIS and mNIS was 0.59 (95% CI, 0.40-0.78); Stuart's Tau-c was 0.45 (95% CI, 0.30-0.61).

Expression of NIS mRNA by molecular subtype

The characteristics of the patients from publicly available datasets included in the analysis of NIS mRNA expression by breast cancer subtype are provided in **Table 5**.

Expression of the NIS gene was higher in local ER- tumors (median 0.053; mean +/- SD=0.047 +/- 0.524) than local ER+ tumors (median-0.027; mean +/- SD =-0.024 +/- 0.544) (**Figure 2a**: Mann-Whitney p=0.013).

When tumors were divided into ER-/HER2-, HER2+, and ER+/

Table 3 Prevalence of cNIS/+/ and mNIS/+/ by HR status, stratified by Her2/neu status (N=90).

		ER/+/ (N=41)		ER/-/ (N=49)		P-value
		N		N		
Her2/ neu negative (N=74)	cNIS					
	/-/	26	79%	14	34%	<0.01
	+	7	21%	27	66%	
	mNIS					
	/-/	30	91%	23	56%	<0.01
	+	3	9%	18	44%	
Her2/ neu positive (N=16)	cNIS					
	/-/	7	88%	2	25%	0.04
	+	1	13%	6	75%	
	mNIS					
	/-/	8	100%	4	50%	0.08
	+	-	-	4	50%	
Total (N=90)	cNIS					
	/-/	33	80%	16	33%	<0.01
	+	8	20%	33	67%	
	mNIS					<0.01
	/-/	38	93%	27	55%	
	+	3	7%	22	45%	

Table 4 cNIS versus mNIS (N=90).

	mNIS				
cNIS	**/-/**	**1+**	**2+**	**3+**	**Total**
/-/	48	-	-	1	49
1+	7	1	-	3	11
2+	5	2	2	3	12
3+	5	3	4	6	18
Total	65	6	6	13	90

HER2- subtypes by the microarray classification, NIS mRNA expression was not different across the molecular subtypes (**Figure 2b**: Kruskal-Wallis *p*=0.141).

Based on the observation that ER-/HER2+ were more likely to demonstrate NIS immunostaining than ER+/HER2- tumors, we hypothesized that NIS mRNA expression would be higher in ER-/HER2- tumors defined by microarray. HER2+ tumors identified by microarray demonstrate a continuous distribution of an estrogen receptor signalling gene expression module in between ER-/HER2- and ER+/HER2- tumors [9]. HER2+ tumors cannot be reliably categorized into ER+/HER2+ and ER-/HER2+ tumors by microarray analysis. Subdivision of the HER2+ subtype according to local ER-testing demonstrated a trend towards higher NIS expression in ER-/HER2+ tumors as compared to ER+/HER2+ tumors (**Figure 2c**: Mann-Whitney p=0.082).

Similarly, expression of NIS was greater in the combined subset of ER-/HER2- and ER-/HER2+ tumors as compared with the combined subset of ER+/HER2- and ER+/HER2+ tumors (**Figure 2d**: Mann-Whitney *p*=0.011).

Discussion

To date, NIS expression and/or activity in human breast cancer has been detected by RT-PCR [10,11], RNase protection assay

Table 5 Characteristics of the publicly available gene expression datasets.

	All	ER-/ HER2-	HER2+	ER+/HER2-	p-value
Total	3091	530	474	2085	
	percentage (%)				
Age					
≤50	39	44	34	31	p<0.001
>50	52	37	47	57	
unknown	14	19	19	12	
Size					
≤2 cm	44	35	35	49	p<0.001
>2 cm	44	45	47	42	
unknown	12	20	18	9	
Nodal Status					
Negative	58	64	49	58	p<0.001
Positive	31	23	36	32	
unknown	11	13	16	10	
Tumor Grade					
1	14	3	4	18	p<0.001
2	35	13	27	43	
3	32	56	42	23	
unknown	20	28	27	16	
Estogen Receptor					
Positive	73	15	45	93	p<0.001
Negative	24	80	50	5	
unknown	3	5	5	2	
Systemic Therapy					
Untreated	42	50	38	41	p<0.001
Treated	46	36	45	48	
unknown	12	14	17	11	

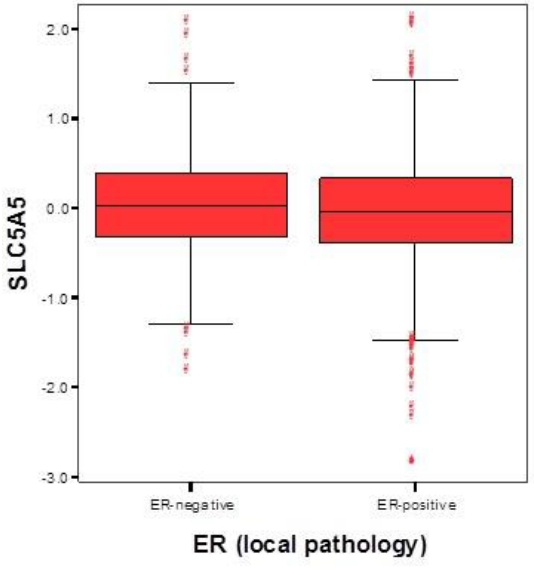

Figure 2a NIS mRNA expression according to local pathology ER-assessment (N=2710, *p* = 0.013).

[12], Western blot [12], scintigraphy [11,13], and most commonly immunohistochemistry [3-5,12-15].

By immunohistochemistry [10,12,14,15], previous studies have detected cell surface (mNIS) and/or predominantly intracellular (cNIS) NIS protein in 80-90% of breast tumors. In patients who had developed metastatic disease [13], the percentage of NIS-positive tumors were reported to be much less frequent, 33-36%. However, these last authors noted -interestingly- that 7 (87.5%) of the 8 NIS-positive cases were ER-negative tumors and that NIS immunostaining was detected in 7/14 (50%) of the ER-negative tumors compared with 1/14 (7%) of ER-positive tumors. These authors suggested so that up-regulation of NIS in malignant cells might be an estrogen-independent event.

Our results definitively confirm that NIS expression in BC is more frequent in ER negative primary breast cancer. Using a series of primary breast cancers with similar histological (high) grade and nodal status, we observed cytoplasmic (67% vs 20%) and membranous (45% vs 7%) NIS staining more frequently in ER-negative tumors compared with ER-positive tumors. ER-negative tumors are thus enriched for NIS expression. These observations are further supported by our finding that the mRNA expression of the NIS gene is increased in ER-negative tumors defined by either local pathological testing or microarray classification compared with ER-positive tumors.

Our study is also the first to examine the influence of HER2 gene amplification on NIS expression. Based on experiments in mouse models, Kogai et al. [16] suggested that HER2 overexpression primary breast cancer may be associated with enhanced NIS expression. Although based on a relatively limited series of HER2+ evaluated for NIS immunostaining, our results suggests that it is not the case. On the other hand, they demonstrate that NIS expression is more strongly associated with ER-negativity rather than HER2 amplification. Similarly, NIS mRNA expression is higher in ER-/HER2+ compared with ER+/HER2+ breast cancers.

It is important to note that most prior studies examining NIS expression have not precisely evaluated its cellular localization. Knostman and colleagues [15] was the only prior study to examine cellular localization, reporting that among tumors reported as NIS positive, only 27% (21% of all tumors examined) had detectable cell surface NIS protein. In our work, membranous NIS expression was seen in 24/41 (60%) patients with cytoplasmic staining (2/8 ER+ and 22/33 ER-); the correlation between cNIS and mNIS is quite large: 0.64.

The localisation of the NIS protein may influence its clinical relevance, with greater functional activity for membrane-associated NIS. According to Beyer et al. [17], only a small subset of NIS-positive tumors {8% (3+) to 29% (2 to 3+) according to their scorings} have sufficient cell surface NIS expression to confer detectable radionuclide uptake activity *in vivo*. If we analyze our cases scored mNIS 3+, only 5% (2/41) of the ER+ tumours and 22% (11/49) of the ER- cases would be then theoretically able to take up iodine and to be in vivo detectable. Only 4 patients had 20 to 30% of their cells mNIS stained (as it is the case for the patient about which the Iodine uptake imaging is showed). Using scintigraphic methods [11,13], only 17-25% of NIS-positive breast

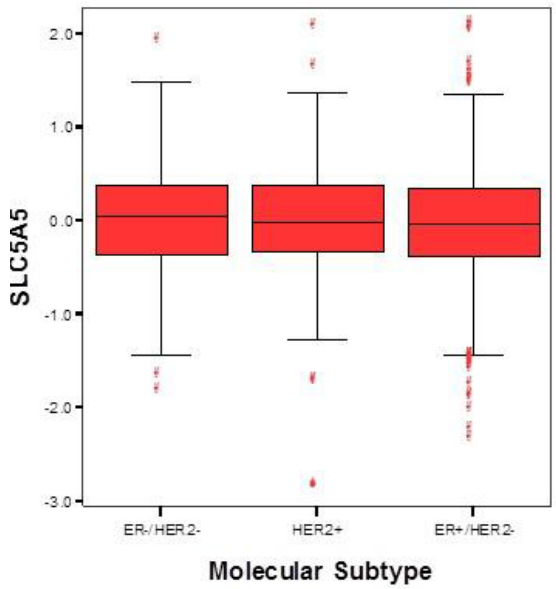

Figure 2b NIS mRNA expression according to microarray based molecular subtype (N=2787, p = 0.141)

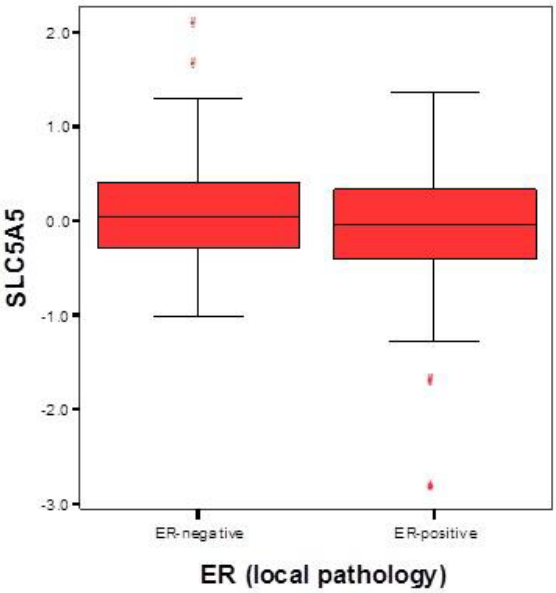

Figure 2c NIS mRNA expression in microarray defined HER2+ subtype according to local ER status (N=392, p = 0.082).

tumors demonstrate detectable radionuclide uptake activity in vivo in their metastases.

Nevertheless, tumors that express only cytoplasmic NIS (20% of the ER positive but 66% of the ER negative) might also benefit from NIS-directed therapies. In a series of malignant thyroid tumors, Castro et al. [18] demonstrated that all NIS immunostained tumors, irrespective of cellular localization and percentages of cells stained, showed radioactive iodide uptake. These thyroid tumors and their metastases were however treated after

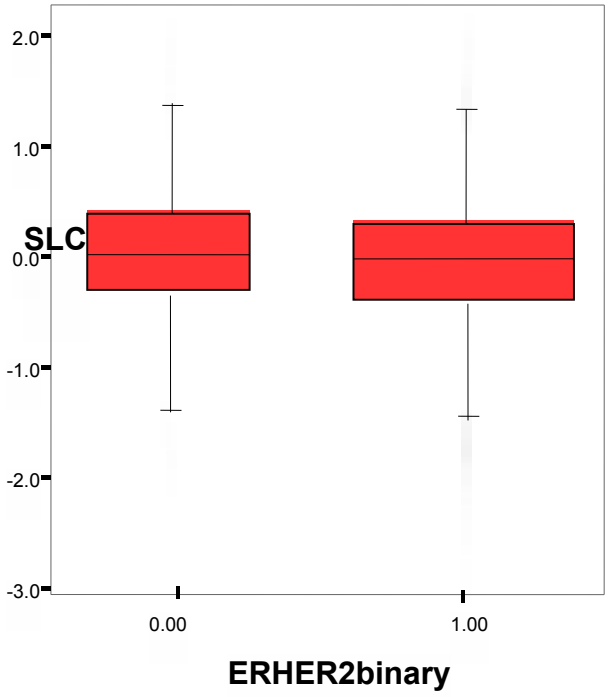

Figure 2d NIS mRNA expression for ER-/HER2- & ER-/HER2+ tumors versus ER+/HER2- & ER+/HER2+ (N=2769, p = 0.011)

thyroid hormone withdrawal and thus under TSH stimulation. This suggests that tumors with cytoplasmic NIS expression may be stimulated to relocalize NIS towards the cell membrane. With regard to BC, prior investigators have demonstrated that NIS expression can be stimulated [19-24]. Arturi et al. [22] demonstrated that the exposure of MCF-7 human breast cancer cell line to insulin, IGF-I, IGF-II, or prolactin induced significant increases in the 125I uptake and in the expression of both NIS mRNA and NIS protein and that in immunocytochemistry studies, NIS was detected mainly in the plasma membrane of the cells. Kogai et al. [19] showed that all-trans retinoic acid (tRA) treatment of MCF-7 cells, an estrogen receptor positive breast cancer cell line, stimulated their iodide uptake in a time- and dose-dependent fashion up to approximately 9.4-fold above baseline and the NIS gene transcription approximately 4-fold.

Additionally, defects in NIS localization in breast cancers may be related to hyperactivation of the PI3K signalling pathway [15] and the IGF-I receptor/PI3K pathway was demonstrated [25] to mediate tRA-stimulated NIS expression in MCF-7, suggesting that pharmacologic PI3K pathway inhibition and radioactive iodine may have additive activity in tumors with cytoplasmic NIS expression.

Conclusion

We have shown that ER negative tumours are more frequently positive for both membranous and cytoplasmic NIS immunostaining than ER-positive cases. Our work suggests that future studies investigating NIS-directed therapies should be enriched with ER-negative breast cancers. We are currently investigating the activity of radioactive iodine therapy in ER-negative breast cancer with detectable NIS expression

Competing Interests

The author(s) declare that they have no competing interests.

Authors' Contributions

PB and DL conceived the study, participated in its design and coordination and drafted the manuscript.

PhB carried out the molecular genetic studies and drafted the manuscript.

DL, BF and NS carried out the analysis of the immunostained slides.

CS and AA participated in the design of the study and helped to draft the manuscript.

LA and MP performed the statistical analysis and drafted the manuscript.

All authors have read and approved the final manuscript.

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