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Analysis Natrium Iodide Symporter Expression in Breast Cancer Subtypes for Radioiodine Therapy Response

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Abstract

Purpose This study investigates natrium iodide symporter (NIS) expression in three breast cancer subtypes to predict radioiodine response.

Materials and Methods Frozen breast tissues from triple negative (TN), human epidermal receptor 2 (HER2+), and luminal A cancers were used in this research. NIS protein expression in each subtype was analyzed using immunohistochemistry (IHC) and western blot (WB). Secondary data such as age, subtypes, and Ki 67 index were drawn from the surgical oncologist database. Breast cancer cell lines were used to investigate the effect of radioiodine by measuring cell proliferation.

Results The forty-one breast cancer samples were analyzed consisted of the following subtypes: TN, HER2+, and luminal A were 58%, 22%, and 20% respectively. The stages of disease were 2A to 4A. Most of samples were at 3B. Ki 67 index of TN, HER2+, and luminal A were 21 ± 12, 19 ± 5, and 7 ± 3 respectively. The NIS expression was detected in 95% of samples in cytoplasm and/or cell membrane; 93% of samples were invasive breast carcinomas. Only 20% of the samples showed NIS expression at cell membrane; four samples were HER2+, and other four were TN subtypes. NIS membrane score was significantly positively correlated with Ki67 index, $p = 0.04$. NIS protein expression was detected at sizes 88 kDa, 50 kDa, and 27 kDa. Cell proliferation rate means of MDA-MB 231, SKBR3, and MCF7 cells were 81.6 ± 4, 10.6 ± 5, and 15.4 ± 13 respectively ($p = 0.009$).

Conclusion NIS protein expression is detectable in breast cancer cells to varying degrees. HER2+ is the most likely to express NIS in the cell membrane followed by TN subtypes. This indicates that radioiodine could be used as a novel adjuvant treatment in breast cancer.

Keywords Cell proliferation · Immunohistochemistry · Ki 67 index · Radioiodine therapy · Western blot

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Introduction

Current breast cancer statistics indicate an upward trend of this disease in the Asia-Pacific region. The age of onset of breast cancer in Asia women's is earlier than that in the West and its mortality rate is higher [1–3]. It is known that identification of the molecular subtypes of breast cancer is crucial to determine the best treatment plan as some of the subtypes are resistant to some therapies [4]. However, some sophisticated treatments that are routinely offered in the developed world for overcoming resistant breast cancer subtypes may be too expensive for most patients in many countries in this region. Therefore, there is a need to developed more economical alternative therapies to improve treatment where financial and medical resources are limited.

Radioiodine has been used for thyroid cancer as adjuvant therapy. It has been known that natrium iodide symporter (NIS) is involved in iodine transport in thyroid and extra-thyroid organs including breast cancer [5, 6]. Therefore, the expression of NIS in breast cancer suggests that there may be a possibility of utilizing targeting radioiodine therapy. The increasing NIS expression by NIS gene therapy using virus vector is effective. However, this therapy method is not easy. Besides, it has safety concerns [7]. Some studies have reported that around 40% of luminal A subtype cancers show resistance to hormone therapy drugs [8, 9]. Human Epidermal Receptor 2+ (HER2+) patients have a lower survival rate and a shorter disease-free interval. This subtype correlates with resistance to conventional therapies [10]. Triple negative (TN) subtype cancer shows an initial good response to chemotherapy but has a poorer prognosis compared to other subtypes [11]. Thus, finding alternative therapy for these subtypes could increase the overall survival rate.

NIS is a transmembrane glycoprotein that contains 13 membrane-spanning segments and three N-linked carbohydrates. It resides in the thyroid in the basolateral membrane of epithelial cells and transports two cations of sodium (Na+) and one anion of iodide (I-) into the cells. This process is facilitated by an enzyme Na+/K+ ATPase [5, 12–14]. In normal thyroid cells, NIS plays a pivotal role in accumulating iodine inside the cells. Its expressions at the membrane of cells allow the application of radioiodine for diagnostic and therapeutic purposes [5, 15].

The presence of NIS has an important role in the effectiveness of this therapy. Radioiodine therapy will be ineffective if the cancer cells do not express NIS. Low NIS expression results in a reduction of radioiodine level uptake in the thyroid [7, 16, 17]. NIS expression has also been reported in non-thyroid tissues such as in salivary glands, gastric mucosa, lactating mammary glands, choroid plexus, ciliary body of the eye, and breast cancer cells [5, 13, 14, 18–21]. The molecular mechanisms of NIS expression in breast cancer remain unclear. However, it is proved that NIS expression influences the radioiodine uptake in breast cancer cells and insertion of NIS genes into the cell membrane of breast cancer cells has been reported to increase radioiodine level uptake followed by an increase the effectiveness of radioiodine therapy [7].

Breast cancer is a heterogeneous disease. According to morphological and molecular observation is classified into at least five subtypes: luminal A, luminal B, HER2+, basal, and normal [4, 11, 13, 22]. However, as far as the authors are aware of there have been no published studies concerning the difference in expression of NIS for different breast cancer subtypes. Even, in South East Asia although certain differences between characteristics of breast cancers in this region with those in the West have already been documented [2, 3].

The purpose of this study is to identify the expression of NIS protein in breast cancer tissue samples based on molecular subtypes to predict the ability of each type of cancer cells to take up radioiodine in vivo. Identification of breast cancer subtypes which express the NIS would help the physician to select which breast cancer patients might benefit from radioiodine therapy when expensive gene insertion procedures are not feasible.

Materials and Methods

The research was held in the Biomedical Laboratory of Andalas University for 8 months (April to December 2016) and The Center of Radioisotope and Radiopharmaceutical Technology, Serpong. The sample tissues were frozen in liquid nitrogen before use. Secondary data such as age, Ki67 index, the subtype of the breast cancer and stages of disease were provided by the Padang branch of the association of surgical oncologists. Stages of disease as classified according to the American Joint Committee on Cancer (AJCC) [4]. Ki67 was used to assess the proliferation index, and an index higher than 14% was considered to be high proliferation [23]. MDA-MB 231, SKBR3 and MCF7 cell lines representing TN, HER2+, and luminal A breast cancer subtypes were used respectively as the subject for in vitro study to measured radioiodine effect. SKBR3 cell line was supplied by the American Type Culture Collection (ATCC). MCF7 and MDA-MB 231 cell lines were gifted from the Faculty of Medicine, Padjadjaran University, Bandung, Indonesia. Ethical approval was obtained from Ethics Committee of Faculty of Medicine of Universitas Andalas.

Immunohistochemistry

The tissue samples were removed from the liquid nitrogen, embedded in paraffin, cut into 4 mm slices and placed on microscope slides. They were then deparaffinized, rehydrated and incubated with sodium iodide symporter antibody (FP5A, Thermo Scientific) at a 1: 200 dilutions for 60 min at room temperature. The slides were rinsed in phosphate buffered

saline (PBS) and incubated in a Starr Trek Universal HRP Detection Kit for 15 min and using a diaminobenzidine (DAB) detection kit. Then, the slides were washed and counterstained with hematoxylin. These steps were performed twice. Histological analysis was observed using an Olympus BX 31 microscope. Thyroid cancer samples were used as positive controls and breast cancer samples incubated without the primary antibody used as negative controls.

The level of NIS expression in the membrane was scored by three experienced breast cancer pathologists using a scale of 0 to 3+ according to HER2+/neu staining criteria. A score of 0 or 1+ was considered negative and a score of 2+ or 3+ was considered a positive result [24]. The cytoplasmic intensity percentage was calculated from the average of three selected fields of view using × 400 magnification. The number of stained cells were counted and compared to all cells in each field of view.

Western Blot

Tissue sample was homogenized in T-PER Reagent (Thermo Scientific 78510) with the ratio of 1:20 (*w/v*), then centrifuged to separate the pellet cell or tissue debris in 4 °C according to the manufacturer's instructions. Protein concentrations were measured. The protein (100 μg) was added to sample buffer (NuPAGE LDS sample buffer 4x, NuPAGE reducing Agent 10x, deionized water) and heated for 10 min at 70 °C. Proteins were then separated by SDS/PAGE (NuPAGE MOPS SDS buffer kit) and transferred to a PVDF membrane (iBlot2 transfer stacks, Thermo). The blot was stained using a pre-stain standard (SeeBlue Plus2, Thermo) to check the transfer of protein on the membrane. To avoid nonspecific binding, a blocking buffer (Staring Block T20, Thermo) was added to the membrane for 30 min. Then, the membrane was incubated with monoclonal antibody Sodium Iodide Symporter (FP5A, Thermo Scientific) 1:1000 at room temperature for one hour, then at 4 °C overnight. After three times washing, the membrane was incubated with secondary antibody 1:200 (goat anti-mouse IgG (H+L), Peroxidase Conjugate, Thermo Scientific) for two hours at room temperature. Next, the membrane was covered with Horseradish peroxidase (1-step ultra TMB-Blotting solution). PVDF membrane was stripped and re-probed with Na^+/K^+ ATPase alpha antibody (M7-PB-E9, Thermo Scientific) as plasma membrane protein markers. Experiments were performed twice.

Determination of Cell Proliferation

An in vitro study was done to investigate the effect of radioiodine by measuring cell proliferation using methylthiazolyldiphenyl-tetrazolium bromide (MTT). MDA-MB 231 and MCF7 cells were cultured in RPMI 1640 and SKBR3 cells in Mccoy's 5A mediums. They were supplemented with 1% FBS in 25 cm^2 culture flasks until 90% confluence, then harvested by trypsinization. The cells were washed with phosphate buffer saline (PBS) twice, then were seeded in a 96-well plate at a density of 2×10^4/well and incubated for 24 h, and treated with 1 μCi (3.7×10^4Bq) Iodine-131 (^{131}I) for 24 h. The control cells were treated with medium only. MTT (5 mg/ml in PBS) was added to the wells. The plate was incubated at 37 °C in the dark for 30 min. The medium was removed and 100 ml dimethyl sulfoxide was added to the wells. The absorbance was measured at 550 nm using the colorimetry. Two experiments were performed in triplicate. The percentage of cell proliferation was calculated by the equation of [25]:

$$\text{Cell Proliferation } \% = \frac{\text{OD test} - \text{OD control}}{\text{OD control}} \times 100$$

Statistical Analysis

Data analysis and results of the study are presented as the means ± standard deviations (SD). Kruskal-Wallis' and Mann-Whitney's tests were used for data analysis, with $p < 0.05$ was considered as statistically significant.

Results

The forty-one breast cancer tissue samples came from patients with an average age of 48 ± 9.3 years old. Subtypes of the cancers were 24 (58%) of TN, 9 (22%) of HER2+, and 8 (20%) of luminal A. Histopathology results of 41 tissue samples showed that most (78%) of it were invasive ductal carcinoma followed by invasive lobular carcinoma (12%). Stages of samples were 2A to 4A with the highest stage was 3B (Table 1). Overall average Ki 67 index was 18 ± 11; 62% of samples were in high proliferation category. The means of Ki 67 index based on TN, HER2+, and luminal A subtypes were 20.5 ± 12, 18.7 ± 5 and 8.6 ± 3 respectively, with $p = 0.001$ (Fig. 1a).

The Expression of NIS

Forty of forty-one samples were examined for expression of NIS. Expression of NIS was observed more frequently in the cytoplasm (95%) than it was in the membrane. The cytoplasm NIS intensity was varied. The average cytoplasm NIS intensity of TN, HER2+, and luminal A were 40.6 ± 23, 51.7 ± 21, and 61 ± 28 respectively, with $p = 0.2$ (Fig. 1b).

Only eight samples that have positive NIS expression results in the membrane; they were all TN or HER2+ subtypes (Fig. 2a, b). Almost half of the HER2+ samples and 17% of TN samples were positive for NIS expression at the

Table 1 Characteristic of the samples based on subtypes of the cancer, histopathology results, and stages of the disease

No.	Subtypes	*N* (%)
1	Triple negative	24 (58%)
2	HER2+	9 (22%)
3	Luminal A	8 (20%)
	Total	4 (100%)
No.	Diagnosis	*N* (%)
1	Invasive ductal	32 (78.0%)
2	Papillary	1 (2.4%)
3	Mucinous and invasive ductal	1 (2.4%)
4	Invasive lobular	5 (12.1%)
5	Mucinous	1 (2.4%)
6	Carcinoma mammae	1 (2.4%)
	Total	4 (100%)
No.	Stages	*N* (%)
1	2A	8 (19.5%)
2	2B	2 (4.9%)
3	3A	4 (9.8%)
4	3B	22 (53.7%)
5	4A	5 (12.1%)
	Total	4 (1005)

membrane. NIS membrane was not detected at luminal A subtype (Fig. 2c). Thyroid tissue (Graves' disease) as positive control (Fig. 2d). Furthermore, the average membrane expression score for HER2+ appeared almost closed to TN, 2 ± 0 and 2 ± 0.5 respectively. These were all invasive breast carcinomas in stage 2 or 3 according to the histology. Furthermore, means of Ki 67 index of positive and negative membrane score were 22.6 ± 12 and 16.6 ± 6, respectively, with $p = 0.008$ (Fig. 3).

Thirty-six of forty-one samples (21 of TN, 8 of HER2+ and 7 of luminal A) have been studied using western blot. The blotting revealed the presence of 88, 50, and 27 kDa bands, which correspond to NIS (Fig. 4a). The protein of NIS is mostly detected at 27 kDa for all of the subtypes. Besides, all samples expressed NIS at the membrane have visible bands at 50 and 27 kDa protein sizes (Fig. 4b).

Cell Proliferation

The proliferation rate was used to analyze the effect of radioiodine in the breast cancer cell lines. Radioiodine exposure reduced proliferation rate until 18.4%, 89.4%, and 84.6% for MDA-MB 231, SKBR3, and MCF7 respectively. Means of cell proliferation after radioiodine exposure were 81.6 ± 4.28, 10.6 ± 5.50, and 15.4 ± 13.63 for MDA-MB 231, SKBR3, and MCF7 respectively, with $p < 0.05$ (Fig. 5).

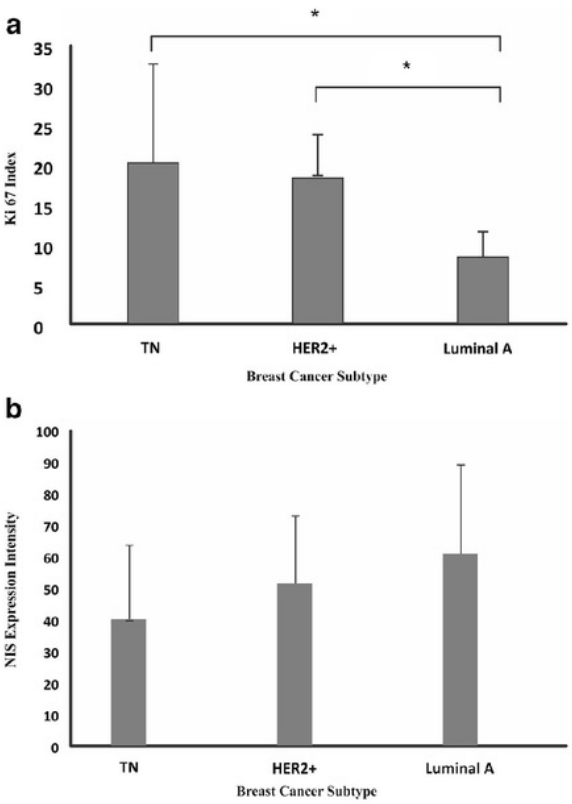

Fig. 1 Molecular markers expression in each breast cancer subtype. **a** The average of Ki 67 index based on the subtype. Asterisk (*) indicates significant difference $p = 0.001$. **b** The intensity of NIS expression in the cytoplasm in various breast cancer subtype

Discussion

Several studies have reported that up to 70–90% of breast cancer samples exhibit NIS expression [18, 21, 26, 27]. In this study, the NIS expression was detected in the cytoplasm, which is 97.5% of the samples with various intensities of staining. This result was similar to another study [20]. A Western blot analysis was performed to determine the level of NIS protein in the samples. It is known that in Graves' disease and COS-7 cells, a fully glycosylated NIS protein with the molecular weight of ~ 90 kDa can be isolated and NIS in human thyroid tissues is 77 kDa [27, 28]. Partially glycosylated NIS protein can have a molecular weight of ~ 50 kDa in tumor diseases and ~ 15 kDa in Graves' and tumor diseases [12, 28, 29]. In this study, Western blot results showed three bands at around 88, 50, and 27 kDa, corresponding to NIS membrane expression with 50 and 27 kDa being most common in HER2+ and TN. Of the 36 samples with available Western blot data, 63% showed that the plasma membrane stained at the 27 kDa band. This finding confirms by the previous studies reporting that in breast tissues, the protein migrates at lower molecular weights [29]. The blotting

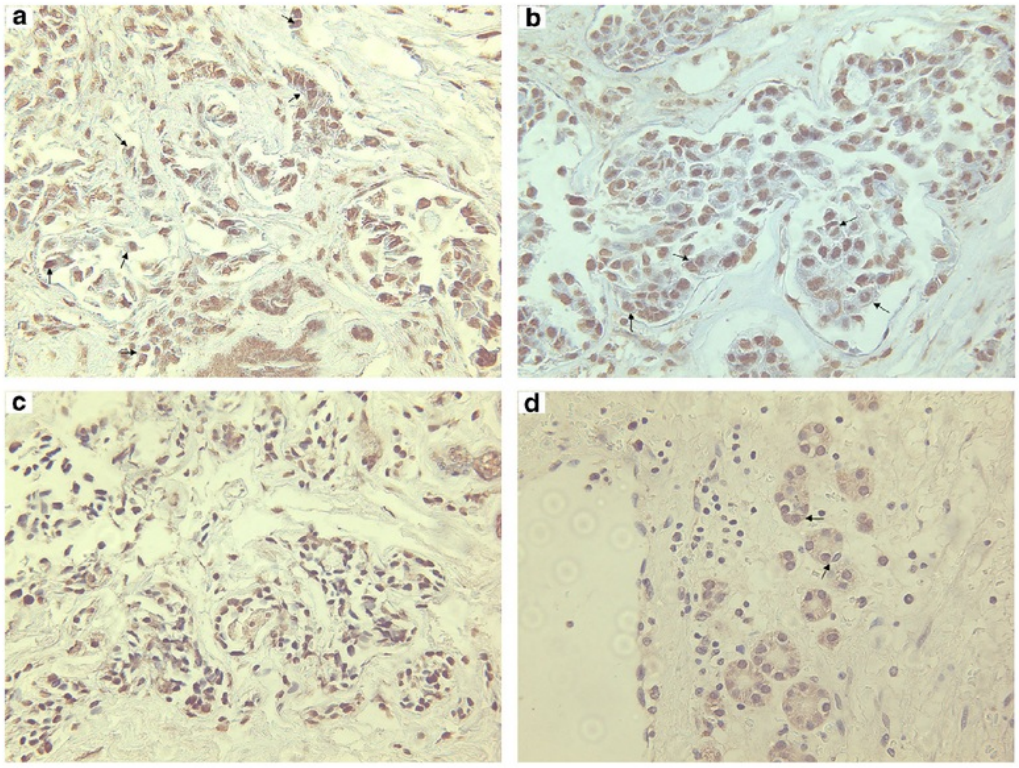

Fig. 2 Immunohistochemistry of NIS expression in membrane from various breast cancer subtypes. **a** TN subtype. **B** HER2+ subtype. **c** Luminal A subtype. **d** Control positive (Graves' disease). Arrows indicate the NIS expression in the membrane. The images are shown in × 400 magnification

revealed the presence of ~ 88 kDa proteins depicting a fully glycosylated of NIS was demonstrated in TN, HER2+, and luminal A subtypes of 67%, 75%, and 57% respectively. The presence of ~ 50 and ~ 27 kDa band partially depicting glycosylated of NIS, and ~ 50 kDa band was demonstrated in TN with 95%, HER2+ with 75%, and luminal A with 71%. A ~ 27 kDa band was demonstrated in TN, HER2+, and luminal A, with 95%, 100%, and 100% respectively. A study reported that radioiodine uptake depends on a fully glycosylated NIS protein [30]. It seems an increasing glycosylation level will be a promising strategy to enhance the role of NIS as radioiodine co-transporter. An in vitro study demonstrated in SKBR3 cell line, ATP, and EGF treatments increases NIS expression at the membrane cell [31]. Further studies are needed to investigate if the treatments are related to glycosylation of NIS or not.

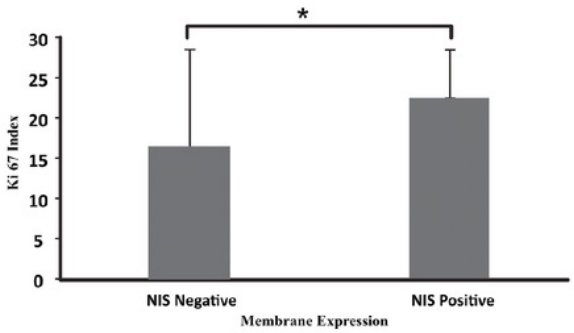

Fig. 3 Comparison of Ki 67 index between samples which positive NIS expressing membrane NIS (+); $N = 8$ and not expressing NIS (−); $N = 33$. Asterisk (*) indicates significant difference ($p < 0.008$)

In this study, 20% of the samples showed that NIS expression at the membranes only observed in HER2+ and TN subtypes. As was found in other breast cancer studies, the membrane expression was less common than cytoplasmic expression [18, 26]. This is in contrast with NIS expression in thyroid cells membrane. In these cells, it is up-regulated by thyroid-stimulating hormone (TSH) [32]. The presence of NIS in normal thyroid tissue was most observed in the membrane cell. In thyroid cancer, NIS is mainly located in the cytoplasm [33]. In this study, means of Ki 67 index of positive membrane score was 22.6 ± 12, while the negative score was 16.6 ± 6, with $p = 0.009$. This finding strengthens the previous reports that found NIS expression increases during malignant transformation [34]. This study did not find any association

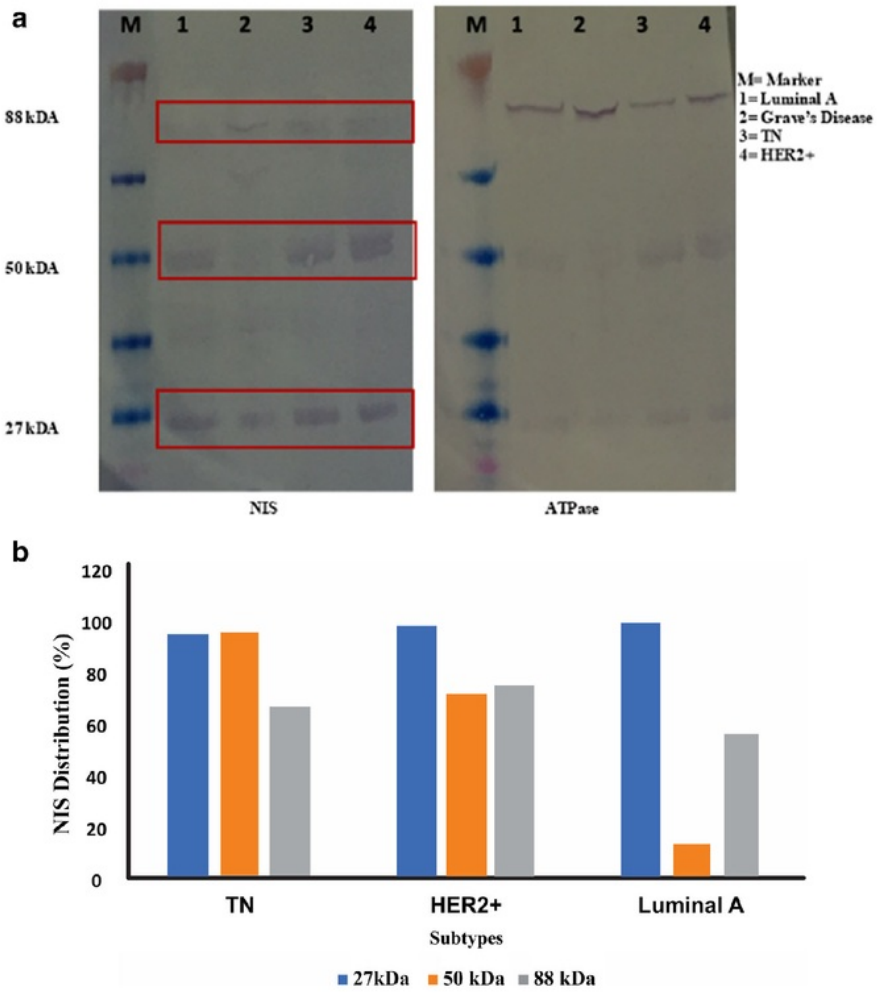

Fig. 4 NIS expression blotting in various breast cancer subtypes. **a** Western blot result. **b** NIS expression distribution in breast cancer subtypes

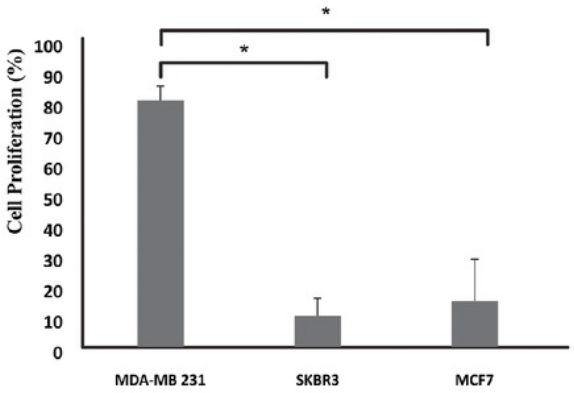

Fig. 5 The proliferation rate of MDA-MB 231, SKBR3, and MCF7 cell lines. ($p < 0.05$). Asterisk (*) indicates significant difference ($p < 0.05$)

between NIS expression in membrane and stages of the disease. This might be due to the absence of early-stage samples.

Nevertheless, a larger number of samples are needed to confirm this finding. If NIS expression correlates significantly with the proliferation index, then NIS expression in breast tissue will respond to cancer therapy quite different from the thyroid. Around 70–80% of thyroid cancers that express NIS are still well-differentiated regardless of their stage [6, 28, 35, 36]. It seems that cell differentiation in thyroid cancer may associate with the NIS expression and radioiodine accumulation [6]. Undifferentiated thyroid cancer is unable to take up radioiodine. This condition is assumed to be due to the absence of or low NIS expression [17, 29, 36]. Poor differentiation of thyroid cancer will reduce NIS expression. This is in contrast with the behavior of breast cancer tissue that found in this study; NIS expression appears to increase parallel with the progress of cell proliferation in breast cancer.

The HER2+ subtype showed the highest frequency and intensity of NIS expression, with almost half of the samples showed NIS expression in the membrane, followed by TN with about one-sixth. Based on the results of this study, HER2+ subtype may be the most suitable to receive radioiodine. The next suitable one is the TN subtype. For luminal A subtype sample, the NIS expression was only observed in the cytoplasm. However, MCF-7 cell line, which is representative of luminal A subtype, showed a radioiodine response in an in vitro study [37].

Based on the in vitro study, radioiodine exposure significantly reduces cell proliferation of SKBR3 and MCF7 cells compared to that of MDA-MB 231 cell, with $p < 0.05$ (Fig. 5). Radioiodine exposure reduces MCF7 cell proliferation up to 84.6%. On the other hand, many in vitro studies reported that MCF7 cell has a very low NIS expression in the membrane. The enhancement of NIS membrane targeting is needed to receive radioiodine effectively [37, 38]. We assumed that NIS membrane expression might be needed for radioiodine to be taken up. However, it seems that the effect of radioiodine does not solely depend on the count of radioiodine inside the cell. The toxicity in MCF7 cell would be due to the indirect effect of β-radiation of radioiodine, resulting in cell death as a bystander effect. It will alter the dynamic equilibrium between proliferation, apoptosis, quiescence or cell differentiation [39–41]. Interestingly, radioiodine exposure in HaCaT cell line almost does not affect cell proliferation [37]. It seems each cell has its sensitivity characterization toward radioiodine exposure. Further studies are needed to confirm this.

The findings of the study suggest that radioiodine therapy would be more likely to be effective for HER2+ and TN subtypes with high cell proliferation rate, as well as luminal A. The relatively high expression of NIS detected in these cancers strongly suggests that radioiodine could be well successfully taken up and slowing cancer growth when other treatments are no longer helpful.

Conclusions

This study suggests that determination of molecular breast cancer subtypes will help identify whether cancer cells might express NIS. HER2+ subtype is most likely to express NIS, both in the cytoplasm and at the cell membrane. This indicates that radioiodine could be used as a novel adjuvant treatment in breast cancer management for these cancer subtypes as it is for thyroid cancer, especially in patients who are unresponsive to the existing treatments. However, the in vivo investigation will be necessary to determine the efficacy of this therapy.

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Compliance with Ethical Standards

Conflict of Interest Aisyah Elliyanti, Dewi Rusnita, Nita Afriani, Yayi Dwina Billianti Susanto, Veronica Y Susilo, Sri Setiyowati, and Wirsma Arif Harahap declare no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent The institutional review board of our institute approved this study and the requirements to obtain informed consent were waived.

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