Original paper

The significance of StAR-related lipid transfer protein-3 expression in breast cancer

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StAR-related lipid transfer domain containing proteins (STARD3) are a group of proteins that contain a steroidogenic acute regulatory protein-related lipid transfer domain. Breast cancer (BC) has been linked to the STARD3 gene. In this study, we sought to confirm the relationship of STARD3 protein expression with clinicopathological characteristics and BC molecular subtypes.

Using tissue microarray, we examined the STARD3 protein expression in 200 BC tissues and 20 normal breast tissues.

Higher protein expression of STARD3 was found in tumor tissues than normal tissues. One hundred and fifty-two (69.1%) of the 200 cases tested positive for STARD3 (high H-score), while seventy (30.9%) had a low STARD3 H-score. When STARD3 is present, its expression ranges from mild to strong. STARD3 H-score was strongly linked to human epidermal growth factor receptor 2 (HER2)-positive ($p < 0.001$) and estrogen receptor (ER)-positive ($p < 0.009$) patients, but not to triple-negative BC patients.

STARD3 high H-score was associated with histological grade and tumor size. No significant associations were found with stage of the disease, pathological stage or node status. Our research revealed that STARD3 levels were higher in tissues from malignant BC, and it was associated with HER2 and ER, suggesting that it might be utilized as a marker for BC.

Key words: breast cancer, STARD3, immunohistochemistry, HER2.

Introduction

The most frequent type of cancer in females is breast cancer (BC), which accounts for almost 25–30% of new cancer diagnoses among females worldwide [1]. Breast cancer is also the leading cause of cancer death in women aged 20–60. Breast cancer is divided into five molecular subgroups based on the 2015 St Gallen International expert consensus definition, each with distinct prognosis and treatment implications: luminal A [estrogen receptor (ER)-positive and/or progesterone (PgR)-positive, human epidermal growth factor receptor 2 (HER2)-negative]; luminal B – HER2-negative (ER-positive and/or PgR-positive, HER2-negative); luminal B-HER2-positive (ER-positive and/or PgR-positive, HER2-positive); HER2-enriched (ER-negative and PgR-negative, HER2-positive); and triple-negative breast cancer (TNBC) (ER-negative and PgR-negative and HER2-negative) [2, 3]. In around one-fifth of all breast tumors, overexpression of the receptor tyrosine kinase HER2 is induced by 17q12–q21 amplification [4]. Cancer progression and aggressive behavior have been linked to HER2-independent signaling pathways, which could explain why HER2-targeted therapy has failed in a large percentage of patients [5, 6].

StAR-related lipid transfer protein 3 (STARD3), also known as metastatic lymph node 64 (MLN64),

is a member of the START domain protein family of lipid transfer proteins [7]. It is located in the q12–21 region of chromosome 17. There are two domains for STARD3: the MENTAL domain binds the protein in late endosomes, while the cytoplasmic START domain facilitates cholesterol transport [7–9]. As a result of this interaction, a membrane contact point between late endosomes and the ER is formed, allowing cholesterol to be transferred to late endosomes.

A recent study revealed that STARD3 may be a target for age-related macular degeneration as it enhances the elimination of intracellular cholesterol and slows the course of the disease [10]. StAR-related lipid transfer protein-3, like other STAR family members, may mediate cholesterol trafficking between late endosomes and other organelles such the endoplasmic reticulum or plasma membrane [11–13]. Overexpression of STARD3 has been shown to improve cholesterol production by boosting the expression of cholesterol synthesis enzymes [13].

StAR-related lipid transfer protein-3 was shown to be highly expressed in HER2-positive breast carcinomas at the mRNA level in 14 out of 19 primary invasive BC cases [14]. Co-silencing of STARD3 with HER2 has been reported to have an additive effect on inhibition of cell proliferation and induction of apoptosis [15]. Previous studies have examined the expression of STARD3 at the mRNA level in BC [13, 16]; STARD3 mRNA level was high in HER2-positive BC and low expression has been detected in ER-positive and TNBC. In BC, especially HER2-positive BC, increased STARD3 expression was associated with lower overall survival (OS), relapse-free survival (RFS), and disease metastasis-free survival (DMFS) [16]. In this study, we aimed to confirm the correlation of STARD3 expression at the protein level in BC, and correlate the expression with clinicopathological features of BC patients using tissue microarray.

Materials and methods

Reagents and tissue microarrays

We used two tissue microarrays (TMA) provided from, US-Biomax; BC081116d and BC081120f. Primary antibody against STARD3 was purchased from Abcam [anti-MLN64 antibody (ab3478)]. The Mouse and Rabbit Specific HRP/DAB immunohistochemistry (IHC) Detection Kit was purchased from Abcam (cat number: ab64261).

Case selection

The two TMAs contain 200 cases of invasive breast carcinomas and 20 normal breast epithelial tissues. There were 200 core biopsies from 200 patients and 20 cores from normal tissues. The core diameter was 1 mm and the thickness was 5 μm. There were no special types of invasive breast carcinoma. Specialized pathologists reevaluated the original hematoxylin and eosin slides and all of the special staining results. The clinicopathological medical records were collected: age, sex, tumor size, histological grade, pathological stage, lymph node status and BC molecular subtypes, based on the 2018 Tumor Node-Metastasis Staging System developed by the American Joint Committee on Cancer. As shown in Table I, tumor size (T) was classified into four categories according to the TNM grading system: T1 – size $\leq$ 2 cm (n = 15 cases), T2 – size $>$ 2 cm but $\leq$ 5 cm (n = 137 cases), T3 – size $>$ 5 (n = 23) and T4 – with skin involvement and inflammatory carcinoma (n = 25 cases). The lymph node status was determined using the following criteria: N0 – no regional lymph node metastasis (n = 135 cases), N1 – metastasis in 1–3 regional lymph nodes (n = 48 cases) and N2 – metastasis in 4–9 regional lymph nodes (n = 17 cases). There were no distant metastases in any of the cases. The tumor grade was classified into three groups: well-differentiated (n = 57), moderately differentiated (n = 133) and poorly differentiated (n = 7). Furthermore, the clinical stage system was classified as follows: Stage I (n = 15 cases), Stage II (n = 141 cases), Stage III (n = 44 cases).

Immunohistochemical staining and evaluation

To determine the clinical importance of STARD3 expression in BC, immunohistochemical staining for STARD3 was done on TMA BC tissues, which included a total of 220 cases. For antigen retrieval, the TMA slides were pretreated by heating at 95°C for 30 min in a citric acid retrieval buffer (pH = 6.0). Then, the sections were incubated with 3% H_2O_2 for 10 min to block endogenous peroxidase. The slides were incubated with antibody raised against STARD3 (1 : 200 dilution) in a humidified chamber at 4°C overnight. The immunohistochemical staining was established according to the Mouse and Rabbit Specific HRP/DAB IHC Detection Kit. Specialized pathologists evaluated STARD3 immunostaining. Staining was evaluated based on the intensity and the percentage of positively stained tumor cells. If the cytoplasm and nuclei of the tumor cells were at least faintly stained with STARD3, the cells were classified as STARD3-positive. The H-score was calculated using the following equation: H-score = $Pi\,(i + 1)$, where i is the intensity of the stained tumor cells (0–3+), and Pi is the percentage of stained tumor cells for each intensity [17]. The cutoff value was set as 6 out of 12, which was the mean of the H-score. Cases that exceeded H-score = 6 were considered as having high STARD3 expression. For a positive control we used adrenal gland tissues (pheochromocytoma) and IgG as a negative control.

Statistical analysis

SPSS version 18.0 was used to analyze the data (SPSS, Inc., Chicago, IL, USA). One-way ANOVA and χ^2 tests were used to analyze the association between STARD3 and BC clinicopathological parameters. Statistical significance was defined as * $p < 0.05$.

Results

Patients' characteristics

The clinicopathological characteristics of the 200 BC patients studied in this research are presented in Table I. In this study we used two microarrays, 200 invasive BC tissue cases, and 20 cores were normal breast tissues. About 129 of the patients were over 45 years old, whereas 91 were under 45 years old. At the time of diagnosis, three of the patients were under the age of 30. There were 58 HER2-positive cases and 142 HER2-negative cases, 116 ER-positive cases and 84 ER-negative cases, 46 TNBC cases and 154 non-TNBC cases.

StAR-related lipid transfer protein-3 positivity and clinicopathological features

The breast cancer cases were evaluated according to expression of STARD3 (negative, weak 1+, moderate 2+ and strong 3+); representative images are shown in Figure 1. The patients were divided into two groups: high H-score STARD3 and low H-score (Fig. 2 A). One hundred fifty-two out of 220 (69.1%) cases were positive for STARD3 (high H-score) and 70 out of 220 (30.9%) cases had a low STARD3 H-score (Table II). The H-score for STARD3 ranged from 0 to 12 with an average of 8.9. The details of STARD3 H-score with clinicopathological parameters are shown in Table II.

StAR-related lipid transfer protein-3 immunoexpression was found to be higher in tumor tissues ($n = 200$) with H-score = 10.4 than in normal tissues ($n = 20$) with H-score = 7.7; p-value = 0.043. StAR-related lipid transfer protein-3 was associated with tumor grade; high STARD3 H-score tended to be more associated with moderately (99/155) to poorly differentiated (41/155) tumors in comparison to well-differentiated tumors. High SATDR3 H-score was more associated with patients older than 45 years (95/150) than patients under 45 years old. StAR-related lipid transfer protein-3 was also found to be associated with larger tumor size ($p < 0.001$), advance tumor stage ($p < 0.001$), HER2-positive ($p < 0.001$), ER-positive ($p < 0.001$), and non-TNBC patients ($p < 0.01$). In ER-positive BC cases ($n = 79$; average H-score = 10.2), the STARD3 H-score was higher than in ER-negative BC cases ($n = 62$; average H-score = 9.2) with p-value = 0.009. Furthermore, the STARD3 H-score was higher in HER2-positive BC cases ($n = 55$; average H-score = 10.7) than in HER2-negative BC cases ($n = 156$; average H-score = 8.7), with a p-value of less than 0.001.

Table I. Clinicopathological features of 220 breast cancer cases

Clinicopathological characteristics	Parameters	n (%)
Age	More than 45	91 (41)
	Less than 45	129 (59)
Histological grade	Well-differentiated	57 (28.5)
	Moderately differentiated	133 (66.5)
	Poorly differentiated	7 (3.5)
	†NA	3
Tumor size	T1	15 (7.5)
	T2	137 (68.5)
	T3	23 (11.5)
	T4	25 (12.5)
Tumor stage	Stage I	15 (7.5)
	Stage II	141 (70.5)
	Stage III	44 (22)
	Stage IV	0
Node	N0	135 (67.5)
	N1	48 (24)
	N2	17 (8.5)
HER2††	HER2-positive	58 (29)
	HER2-negative	142 (71)
ER††	ER-positive	116 (58)
	ER-negative	84 (42)
TNBC††	TNBC	46 (23)
	Non-TNBC	154 (77)

ER – estrogen receptor, HER2 – human epidermal growth factor2, TNBC – triple-negative BC, †NA not available/††Number of cases = 200 excluding normal tissues

When STARD3 was present, the H-score tended to be moderate to strong. In 20 cases, STARD3 staining was patchy, with less than 50% of them being TNBC patients. When it came to HER2 and ER-positive cases, more than 50% of the staining was spotty. StAR-related lipid transfer protein-3 protein expression has been detected in the cytoplasm and nuclear of the BC cells (Fig. 2 B).

Discussion

According to a prior study on the chr17q copy number (CN) trends for HER2 and HER2-related genes. *HER2* gene is localized at chr17q12, one of the adjacent genes to *HER2* that shown high CN concurrently with *HER2* is *STARD3*, which is located at ch17q21 [18]. The StAR-related lipid transfer protein-3 and *HER2* genes are located 30–40 kb apart on chromosome 17q11–12. There are several genes in this region. Coamplification of *STARD3* with *HER2* has been identified because of the strong interaction between these two genes. It is still unknown wheth-

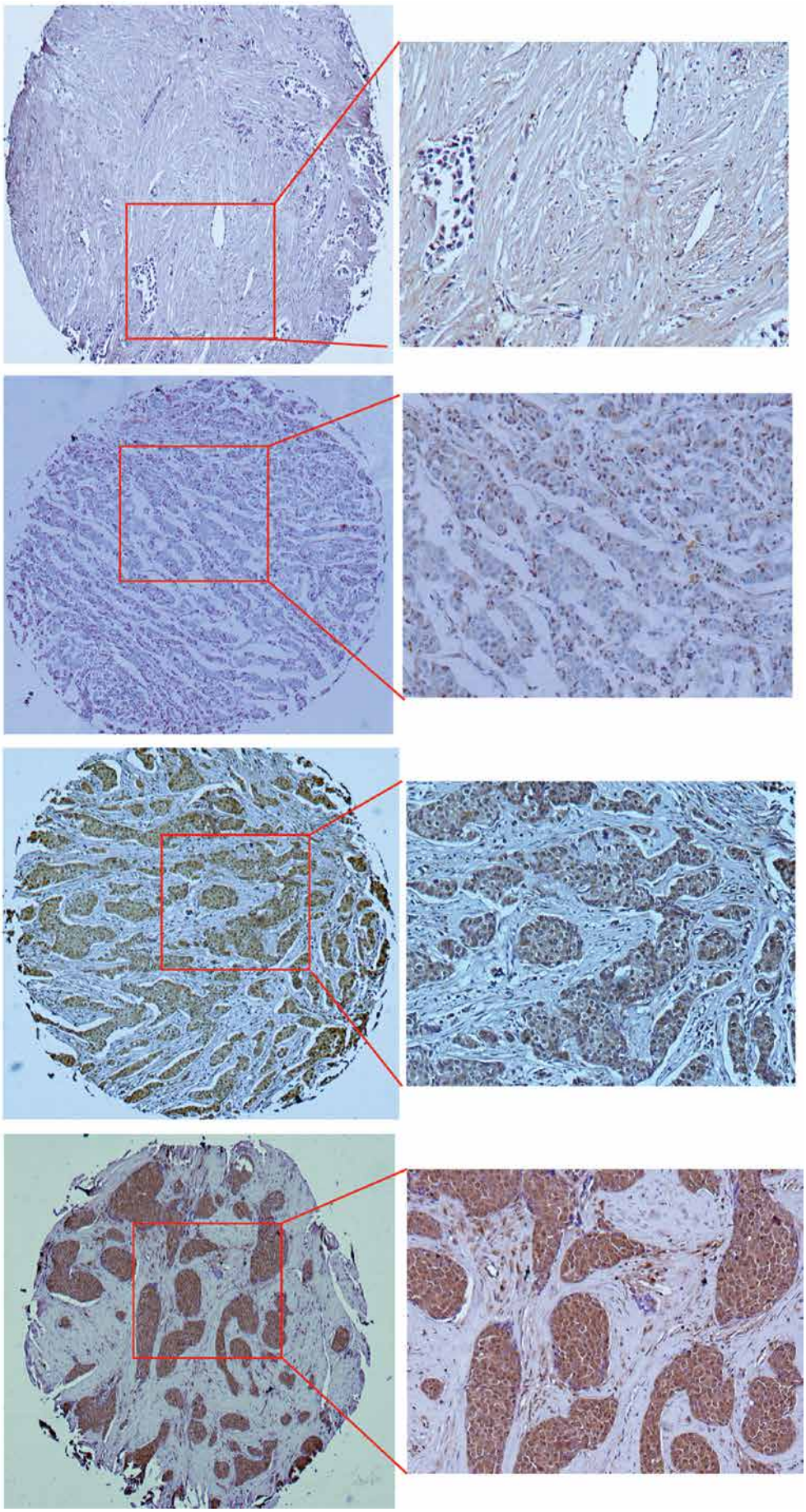

Fig. 1. Representative figure depicting the StAR-related lipid transfer protein-3 immunohistochemistry scoring system (original magnification 40× and 100×). The scoring system was determined as negative, weak (1+), moderate (2+), and strong (3+) based on SATRD3 staining intensity

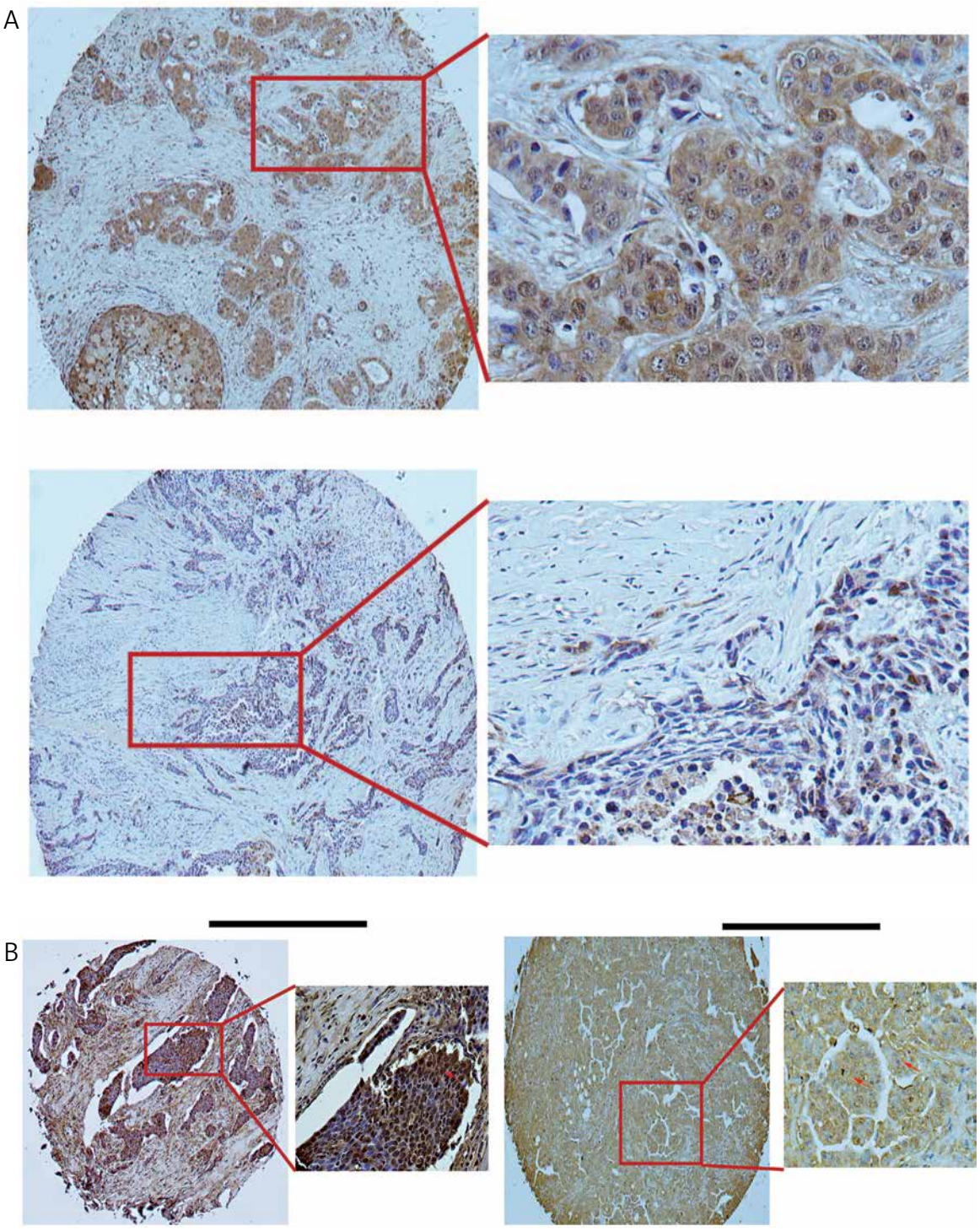

Fig. 2. Representative images for low H-score (A) vs. high H-score (B). Cytoplasmic and nuclear staining for StAR-related lipid transfer protein-3 in tumor cells (original magnification 40× and 200×)

er this amplification could lead to STARD3 overexpression and subsequently BC carcinogenesis. Few studies have been conducted to unveil the molecular role of STARD3 in HER2-positive BC.

Survival of HER2-amplified cells has been linked to STARD3. Indeed, RNA interference inhibits *STARD3* expression solely in HER2-amplified cells, limiting cell proliferation [15]. A previous study demonstrated that epigenetic changes on the *STARD3* gene such as DNA methylation did not play a role in the *STARD3* overexpression in BC. However, promoter analysis of both *STARD3* and *HER2* genes has shown to share the *SP1* transcription factor binding site [7]. As a result, they concluded that a member of the Sp/KLF family may mediate *STARD3* and *HER2* gene expression in cancers.

Table II. Clinicopathological features of breast cancer with high vs. low STARD3 expression based on H-score, $n = 200$ invasive breast carcinomas

Clinicopathological characteristics	Parameters	Low STARD3 n/total	High STARD3 n/total	*p*-value
Age	Less than 45	31/70	50/150	0.051
	More than 45	33/70	86/150	
Histological grade	Well-differentiated	3/45	5/155	< 0.001*
	Moderately differentiated	26/45	99/155	
	Poorly differentiated	16/45	41/155	
	†NA	0	10/155	
Tumor size	T1	7/45	8/155	0.042*
	T2	32/45	105/155	
	T3	2/45	21/155	
	T4	4/45	21/155	
Tumor stage	Stage I	7/45	8/155	0.066
	Stage II	29/45	112/155	
	Stage III	9/45	35/155	
Node	N0	29/45	106/155	0.887
	N1	12/45	36/155	
	N2	4/45	13/155	
HER2	HER2-positive	6/65	49/155	< 0.001*
	HER2-negative	60/65	96/155	
	NA	1/67	8/155	
ER	ER-positive	13/35	66/106	0.009*
	ER-negative	20/35	40/106	
	NA	0	81/106	
TNBC	Non-TNBC	19/59	145/161	< 0.001*
	TNBC	26/59	10/161	

*ER – estrogen receptor, HER2 – human epidermal growth factor 2, TNBC – triple-negative BC, †NA – not available. Clinicopathological parameters were assessed using χ^2 analysis, *$p < 0.05$*

StAR-related lipid transfer protein-3 has been linked to the development of a variety of malignancies, including colorectal, prostate and gastric cancers [19–22]. StAR-related lipid transfer protein-3 was recently found to be overexpressed at the mRNA level in BC, notably in HER2-positive BC, and to be low in ER-positive and TNBC patients. In fact, roughly a quarter of all BC cases show co-overexpression of *HER2* and *STARD3*, as we point above [23].

To confirm the association between STARD3 and BC molecular subtypes, we investigated the expression of STARD3 at the protein level in BC using IHC assay. We looked at STARD3 protein expression in 200 cases of BC, with 116 ER-positive, 58 HER-positive, and 46 TNBC cases. As we expected, STARD3 H-score was highly linked with HER2-positive and ER-positive, but not with TNBC. This is consistent with a previous study that reported increased *STARD3* mRNA expression in the ER-positive cases, as demonstrated by the MCF7 cell line [24].

Patients with high STARD3 levels typically experienced poor clinical outcomes and had shorter OS, RFS, and DMFS compared to those with lower levels, based on previous research [21, 24]. In our study, we found a link between high STARD3 protein expression and tumor grade and tumor size, but not with clinical stage or node status, which is consistent with the role of STARD3 in tumor proliferation.

In contrast to our work, a recent large study examined STARD3 protein expression by IHC in over 2000 human BC cases from two complete Finish cohorts [13] using anti-STARD3 affinity-purified antibody. The results showed that just 10% of BC had strong STARD3 expression. STARD3 is hypothesized to be involved in BC carcinogenesis via cholesterol metabolism disruption and changing the makeup of lipids in cellular membranes. A previous study demonstrated that overexpression of STARD3 enhances cholesterol biosynthesis and increases the SRC kinase activity in BC cell lines. Thus, it may contribute to BC tumorigenesis by enhancing the signaling pathways [13].

Moreover, fusion genes between STARD3 and PPP1R1B have been detected in around 4 cases out of 18 primary gastric cancer tissues, which may play an important role in gastric cancer development through PI3K/AKT [22]. In BC, no research has been done to elucidate the chimeric fusion genes that comprise STARD3.

An intriguing result revealed that the lack of STARD3 expression in preadipocytes, as revealed by

3T3-L1 cell lines, resulted in decreased reactive oxygen species (ROS) production from mitochondria, as well as inhibiting adipocyte differentiation [25]. It is critical to elucidate how STARD3 affects ROS levels in BC and therefore affects BC development.

A few points need to be mentioned; firstly, it has been demonstrated that cases with ER positivity also exhibited high levels of STARD3 that were unrelated to HER2 amplicon overexpression. The molecular basis for the link between ER and STARD3 has not yet been fully elucidated. Second, according to Figure 2 B, STARD3 protein expression is primarily found in the cytoplasm and in the nuclei of some cells. To ascertain whether STARD3 has a role in the nucleus, more studies are required. Finally, additional research is required to compare STARD3 protein expression across cohort studies in various fields.

Additionally, we found some heterogeneity in normal tissues, with most having weak to moderate STARD3 levels and only a few having significant levels. It could be due to the tiny section size in compared to the whole section; this can be considered as a limitation of using tissue microarrays.

Conclusions

Our IHC investigation revealed that malignant BC tissues had higher levels of STARD3 immunoexpression than normal tissues. Poor prognostic variables such as tumor size and histological grade have been linked to high STARD3 H-score. StAR-related lipid transfer protein-3 has a strong correlation with HER2-positive BC, suggesting that it may serve as a marker for this type of cancer.

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The authors declare no conflicts of interest.

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