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PRECLINICAL STUDY

Low RUFY3 expression level is associated with lymph node metastasis in older women with invasive breast cancer

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Abstract

Purpose Sentinel lymph node biopsy is omitted in older women (≥ 70 years old) with clinical lymph node (LN)-negative hormone receptor-positive breast cancer as it does not influence adjuvant treatment decision-making. However, older women are heterogeneous in frailty while the chance of recurrence increase with improving longevity. Therefore, a biomarker that identifies LN metastasis may facilitate treatment decision-making. *RUFY3* is associated with cancer progression. We evaluated *RUFY3* expression level as a biomarker for LN-positive breast cancer in older women.

Methods Clinical and transcriptomic data of breast cancer patients were obtained from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC, $n = 1903$) and The Cancer Genome Atlas (TCGA, $n = 1046$) Pan-cancer study cohorts.

Results A total of 510 (METABRIC) and 211 (TCGA) older women were identified. LN-positive breast cancer, which represented 51.4% (METABRIC) and 48.4% (TCGA), demonstrated worse disease-free, disease-specific, and overall survival. *RUFY3* levels were significantly lower in LN-positive tumors regardless of age. The area under the curve for the receiver operator characteristic (AUC-ROC) curves showed *RUFY3*-predicted LN metastasis. Low *RUFY3* enriched oxidative phosphorylation, DNA repair, MYC targets, unfolded protein response, and mtorc1 signaling gene sets, was associated with T helper type 1 cell infiltration, and with intratumor heterogeneity and fraction altered. Low *RUFY3* expression was associated with LN-positive breast cancer and with worse disease-specific survival among older women.

Conclusion Older women with breast cancers who had low expression level of RUFY3 were more frequently diagnosed with LN-positive tumors, which translated into worse prognosis.

Keywords RUFY3 · Geriatric oncology · Axillary lymph node · Breast cancer

Fernando A. Angarita and Masanori Oshi contributed equally to this work. Presented at 2021 San Antonio Breast Cancer Symposium, San Antonio, TX, USA, December 7–10, 2021.

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Introduction

Older women [(≥ 70 years old (yo)] account for 30% of all newly diagnosed breast cancer patients [1]. Older women generally have tumors with favorable features, such as small size, clinically node-negative, and hormone receptor (HR)-positive [2], which translate into a more than 98% ten-year breast cancer-specific survival [3]. Consequently, decision-making must be carefully planned out in these women. The patient's breast cancer features should be weighed against other unique features in this age group, including competing comorbidities [4], increased vulnerability to cancer treatment toxicities [5], and treatment goals primarily focused on maximizing quality over quantity of time [6]. Several studies including the Cancer and Leukemia Group B (CALGB) 9343 trial and PRIME II have shown that in older women (≥ 70yo) with HR receptor-positive T1 (≤ 2 cm) breast cancer, disease-free survival (DFS) is not compromised by omitting surgical staging of the axilla, but only if the patient receives adjuvant endocrine therapy [7–10]. Both the Society for Surgical Oncology (SSO) [11] and the American Society for Breast Surgeons (ASBrS) [12] added measures to the Choosing Wisely campaign of the American Board of Internal Medicine Foundation (ABIM) [13] recommending against routine sentinel lymph node biopsy (SLNB) in older women (≥ 70yo) with early-stage HR-positive HER2-negative invasive breast cancer. More recent studies have confirmed the selective use of SLNB in older women with invasive breast cancer [14–17].

Efforts to minimize the use of lymph node (LN) surgery in older women with breast cancer could have substantial value through eliminating the risk of lymphedema, pain, and nerve damage [18]. The data on small clinically LN-negative HR-negative breast cancers cannot be extrapolated to those with larger tumors and to those with HR-negative and/or HER2-positive cancers. Furthermore, all HER2-positive cancers should receive systemic therapy, with limited exceptions. Given that the purpose of LN surgery is primarily to provide prognostic information to guide adjuvant therapy, alternative means of predicting LN involvement could be valuable. Tumor genomic profiling with the 21-gene RT-PCR assay OncotypeDx® provides reliable prognostic information and prediction of chemotherapy effect in women with HR-positive/HER2-negative cancer with both negative nodes and 1–3 positive nodes [19–21]. For larger HR-positive tumors as well as HER2-positive tumors, there is real potential value of a means to predict LN involvement without axillary surgery.

Nomograms to predict LN-positive tumors have been proposed [22–28]. While these tools have a wide range of age groups, they have a particularly small sample of older women. For example, using data from 36,441 older women with HR-positive breast cancer, the Mayo Clinic group developed a clinical predictive model to identify older women at low risk of LN-positive breast cancer with goal of identifying those who most warrant omission of SLNB [22]. There are no studies assessing the use of these algorithms and how they impact use of LN surgery or oncologic outcome.

Developing a robust diagnostic marker that predicts LN metastasis for older women with clinically LN-negative invasive breast cancer is necessary. This information could help establish the need for adjuvant systemic treatment while avoiding the morbidity of surgically staging the axilla. Several biomarkers have been identified as predictors of LN status, but limitations are always inevitable [29]. An in silico translational approach allows measuring intratumor biomarkers when comprehensive gene expression profile is available. We hypothesize that there may be an intratumor biomarker that predicts LN-positive breast cancer in older women.

RUFY3 (RUN and FYVE domain containing 3), also known as Rap2-interacting protein X (RIPX) or single axon-related1 (Singar1), is a 469-amino acid protein. Although RUFY3 is classically known for its role in neuronal development [30, 31], recent studies have evaluated its pathophysiologic role in cancer. This has been supported by data showing its involvement in cell migration [32], actin cytoskeleton dynamics [33], lipid modification [34], membrane trafficking [35], and cell signaling [36, 37]. Several studies have confirmed *RUFY3* is also involved in cancer cell regulation and cellular proliferation [38–42]. These studies showed that tumors with high *RUFY3* levels are associated with more advanced disease and have worse overall survival (OS) compared to patients with normal *RUFY3* levels. To date, there are no data on the role of *RUFY3* in breast cancer. This study aims to evaluate *RUFY3* intratumor expression levels as a predictive biomarker for LN metastasis in older women (≥ 70yo) with invasive breast cancer.

Methods

Clinical and gene expression data of breast cancer cohorts

Institutional review board (IRB) approval at Roswell Park Comprehensive Cancer Center (Buffalo, New York, United States of America) was waived as publicly available deidentified databases were used. The publicly available cBioPortal [43] was accessed to obtain clinical and transcriptome data from breast cancer patient in The Cancer Genome Atlas (TCGA) Pan-Cancer study ($n = 1046$) [44]

and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) [45] study ($n = 1903$). Data of adult females with invasive breast cancer were obtained, including age and LN metastasis status. Race/ethnicity data were not available from either cohort database. The average value was used for the genes with multiple probes. Older women were defined as women ≥ 70yo as proposed by the Breast International Group [46].

Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA) [47] with hallmark gene sets of the Molecular Signatures Database was performed to explore the signaling pathways related to high and low *RUFY3* expression in breast cancer with a similar approach we previously reported [48–60]. Each cohort was divided into high and low groups by the median expression levels of *RUFY3* gene. GSEA is a publicly available software that allows determine whether an a priori-defined set of genes show statistically significant, concordant differences between two biological states (Broad Institute, http://software.broadinstitute.org/gsea/index.jsp). A false discovery rate (FDR) of < 0.25 was considered statistically significant as recommended by the developer of GSEA.

Cytolytic activity score (CYT)

CYT was defined as the sum of expression of granzyme A (GZMA) and perforin (PRF1) as previously described [60–62]. CYT was used to evaluate overall anti-cancer immune cell killing in the tumor microenvironment. The threshold of dichotomization of the CYT high and low groups was determined by the median of the CYT.

Immune cell fraction estimation

The level of tumor-infiltrating immune cells was estimated using the xCell algorithm [63] in a similar fashion we previously reported [48–51, 64–68]. xCell algorithm was used to examine whole-tumor transcriptome to score the relative abundance of 64 types of immune and stromal cells across tumors, as previously described [69–72]. The xCell score for each sample was calculated using R software (version 4.0.1, R Project for Statistical Computing), as previously reported [58, 73–75].

Statistical analysis

Statistical analyses were performed using R software. Groups were compared using Mann–Whitney *U* test. Receiver operating characteristic (ROC) curve with area under the curve (AUC) value was used to evaluate accuracy. Survival was plotted by the Kaplan–Meier method and compared using log-rank test. Statistical significance was set at a $p < 0.05$.

Results

Cohort tumor characteristics

Table 1 summarizes the tumor characteristics of older women in the METABRIC and TCGA cohorts. Of 1903 women in the METABRIC cohort, 510 were older women, of which 51.4% were LN-positive. Of 1046 women in the TCGA cohort, 211 were older women, of whom 48.8% were LN-positive. In the METABRIC cohort, patients with LN-positive breast cancer had significantly higher rates of high-grade tumors compared to LN-negative tumors (43.5% versus 37.1%, $p = 0.04$). The TCGA cohort confirmed that patients with LN-positive breast cancer had significantly higher rates of advanced stage tumors compared to LN-negative tumors (55.3% versus 0.9%, $p < 0.001$). LN involvement was not statistically different according to molecular subtype in neither the METABRIC ($p = 0.2$) nor TCGA cohorts ($p = 0.1$).

Older women with lymph node-positive breast cancer are significantly associated with worse survival

To assess whether LN involvement affected oncologic outcomes, both the METABRIC ($n = 1903$) and TCGA ($n = 1046$) cohorts were queried. Figure 1 compares the DFS, disease-specific survival (DSS), and OS according to LN status in the whole cohort (Fig. 1a) and older women (Fig. 1b). Patients with LN-positive breast cancer had significantly lower DFS, DSS, and OS compared to patients with LN-negative breast cancer (Fig. 1a). LN-positive breast cancer had significantly lower DFS, DSS, and OS compared to LN-negative breast cancer among older women (Fig. 1b).

RUFY3 expression levels are predictive of lymph node involvement in older women with breast cancer

To test whether *RUFY3* predicts LN involvement, *RUFY3* expression levels were measured in women with breast cancer and compared according to LN involvement (Fig. 2). *RUFY3* expression levels were significantly lower among women with LN-positive breast cancer in the METABRIC cohort (all age, $p = 0.002$ and older women $p < 0.001$).

Table 1 Tumor characteristics of older women breast cancer patients in the METABRIC and TCGA cohorts

Variable	LN-negative	LN-positive	*p* value
METABRIC ($n=510$)	$n=248$	$n=262$	
Molecular subtype			
ER+/HER2−	202 (81.4)	225 (85.9)	0.2
TNBC	26 (10.5)	17 (6.5)	
HER2+	20 (8.1)	19 (7.2)	
Unknown	0 (0)	1 (0.4)	
Nottingham grade			
1	29 (11.7)	15 (5.7)	0.04
2	117 (47.2)	120 (45.8)	
3	92 (37.1)	114 (43.5)	
Unknown	10 (4.0)	13 (5.0)	
AJCC stage			
0	1 (0.4)	0 (0)	< 0.001
I	73 (29.4)	0 (0)	
II	81 (32.7)	155 (59.2)	
III	4 (1.6)	27 (10.3)	
IV	2 (0.8)	1 (0.4)	
Unknown	87 (35.1)	79 (30.1)	
TCGA ($n=211$)	$n=108$	$n=103$	
Molecular subtype			
ER+/HER2−	68 (62.9)	56 (54.4)	0.1
TNBC	14 (12.9)	7 (6.8)	
HER2+	16 (14.8)	23 (22.3)	
Unknown	10 (9.3)	17 (16.5)	
T category			
T1	39 (36.1)	18 (17.5)	< 0.001
T2	57 (52.8)	52 (50.5)	
T3	11 (10.2)	22 (21.3)	
T4	1 (0.9)	11 (10.7)	
Unknown	0 (0)	0 (0)	
AJCC stage			
I	39 (36.1)	0 (0)	< 0.001
II	68 (62.9)	40 (38.8)	
III	1 (0.9)	57 (55.3)	
IV	0 (0)	2 (1.9)	
Unknown	0 (0)	4 (3.9)	

AJCC American Joint Committee on Cancer; *ER* estrogen receptor; *HER2* human epidermal growth factor 2; *LN* lymph node; *METABRIC* Molecular Taxonomy of Breast Cancer International Consortium; *TCGA* The Cancer Genome Atlas; *TNBC* triple-negative breast cancer

However, there was no significant difference in the TCGA cohort for either women of all ages ($p=0.7$) or older women ($p=0.1$) (Fig. 2a). ROC curves showing the predictive sensitivity and specificity of *RUFY3* are shown in Fig. 2b. The AUC for the ROC curves showed *RUFY3*-predicted LN involvement (METABRIC cohort: all ages AUC = 0.542 and older AUC = 0.627; TCGA cohort all ages AUC = 0.504 and older AUC = 0.577).

RUFY3 expression level is not associated with pathological grade and molecular subtypes

Table 2 summarizes tumor characteristics of older women with breast cancer according to *RUFY3* expression levels. In both the METABRIC and TCGA cohorts, patients with low and high expression of *RUFY3* had similar age at diagnosis (METABRIC: 75yo versus 76yo, $p=0.7$; TCGA: 75yo versus 77yo, $p=0.07$) and distribution of molecular subtypes (METABRIC $p=0.9$; TCGA $p=0.1$). The METABRIC confirmed *RUFY3* expression level was not associated with pathological grade ($p=0.6$).

Breast cancers with low expression of RUFY3 enriched oxidative phosphorylation, DNA repair, MYC targets v1, unfolded protein response, and mtorc1 signaling

GSEA was used to study the enrichment of hallmark gene sets according to *RUFY3* expression level among older women with breast cancer in the METABRIC and TCGA cohorts (Fig. 3). Older women with tumors that had low expression of *RUFY3* showed enrichment of oxidative phosphorylation, DNA repair, MYC targets v1, unfolded protein response, and mtorc1 signaling genes.

Breast cancers with low expression of RUFY3 are associated with a high fraction of T helper type1 (Th1) and type2 (Th2) cells, Tregs, and M1 macrophages and high level of intratumor heterogeneity and fraction altered

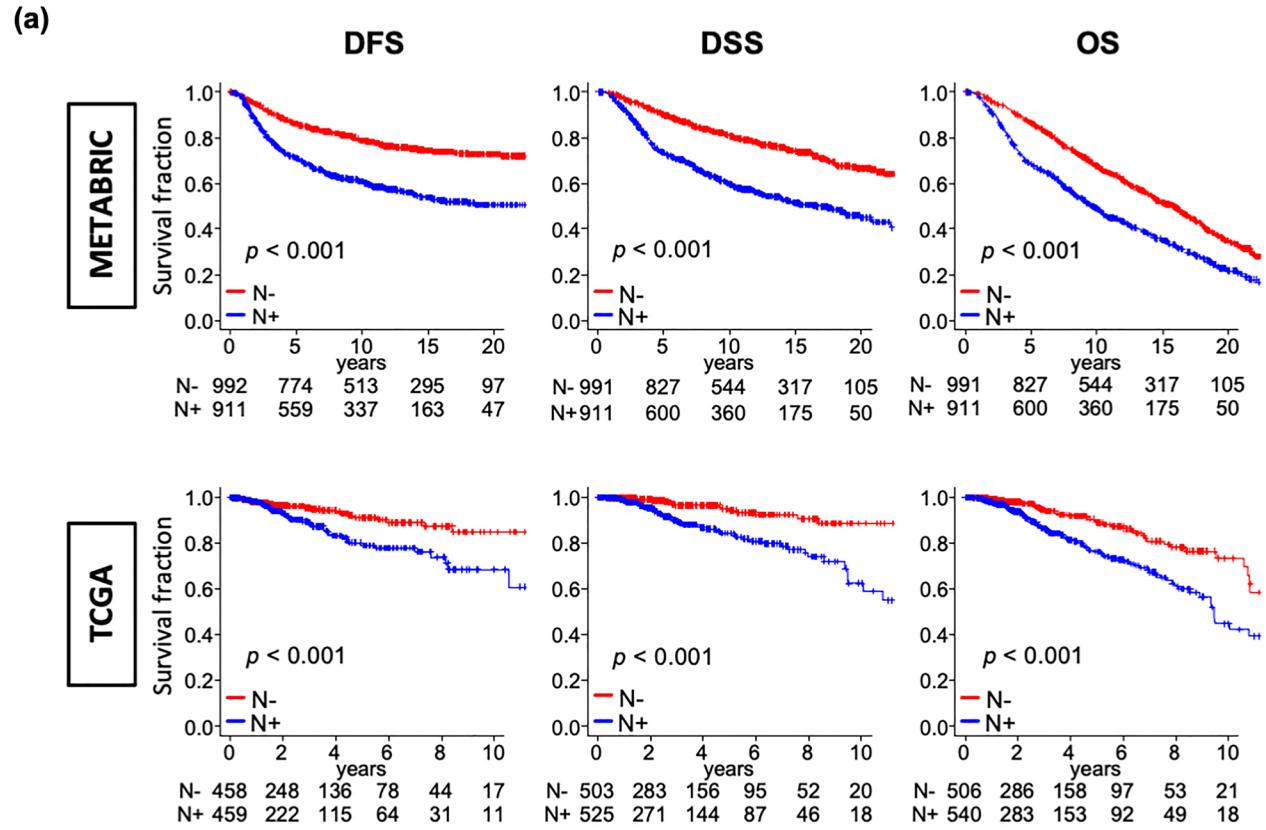
Oncogene c-Myc can induce DNA damage, increase reactive oxygen species, and genetic instability [76], which may lead to elevated mutation load and neoantigens that attract immune cells. Given that breast cancers with low expression of *RUFY3* enriched MYC targets, DNA repair, and oxidative phosphorylation gene sets in the GSEA, it was of interest to assess whether there were immune cells infiltration in the tumor microenvironment. To evaluate this, level of tumor-infiltrating immune cells was estimated using the xCell algorithm. Cohorts of primary breast cancer were used to analyze *RUFY3* expression differences between immune (T cells, B cells, and myeloid cells), tumor, and stromal cells (Fig. 4). Compared to breast cancers with high expression level of *RUFY3*, those with low expression level had a significantly higher fraction of Th1 cells ($p=0.04$) and Th2 cells ($p<0.001$) and low Tregs fractions ($p=0.01$) in the METABRIC cohort (Fig. 4a). In the TCGA cohort, breast cancers with low expression of *RUFY3* had high fraction of Th1 cells ($p=0.04$) and M1 macrophages ($p<0.001$) compared to tumors with high expression level of *RUFY3* (Fig. 4a). CYT did not significantly vary depending on the *RUFY3* expression level in either cohort (Fig. 4b). Breast cancers with low expression of *RUFY3* had significantly higher levels of intratumor heterogeneity ($p=0.001$) and fraction altered ($p=0.005$) compared to tumors with high expression level of *RUYF3* (Fig. 4c).

Breast cancers with low expression of RUFY3 are significantly associated with lymph node involvement and confer worse prognosis particularly in older women

To determine the clinical implication of *RUFY3*, LN involvement was measured by *RUFY3* expression levels in the METABRIC and TCGA cohorts (Fig. 5). In both cohorts, older women with breast cancer that had low expression of *RUFY3* had significantly higher rates of LN involvement, but not in the whole cohorts (Fig. 5a; METABRIC, whole cohort $p=0.002$, older women $p<0.001$; TCGA, whole cohort $p=0.9$, older women $p=0.04$, respectively). The association of *RUFY3* expression levels with survival was assessed by plotting the DSS in the whole population and older women. As shown in Fig. 5b, older women with breast cancers that had low expression of *RUFY3* had significantly worse DSS ($p=0.03$).

Discussion

Given the data reported by other studies on the role of *RUFY3* in cancer [38–42], we assessed the clinical relevance of *RUFY3* as a surrogate for LN metastasis with invasive breast cancer. Women with LN-positive breast cancer had significantly lower expression levels of *RUFY3* compared to LN-negative patients. *RUFY3* expression level was not affected by tumor pathological grade and molecular subtypes. Older women with low expression *RUFY3* tumors enriched oxidative phosphorylation, DNA repair, MYC targets v1, unfolded protein response, and mtorc1 signaling gene sets. The same *RUFY3* low tumors were associated with a high fraction of Th1 and Th2 cells, Tregs, and M1


(a)
DFS
DSS
OS
METABRIC
TCGA
Survival fraction
p < 0.001
N-
N+
years
N- 992 774 513 295 97
N+ 911 559 337 163 47
N- 991 827 544 317 105
N+911 600 360 175 50
N- 991 827 544 317 105
N+ 911 600 360 175 50
N- 458 248 136 78 44 17
N+ 459 222 115 64 31 11
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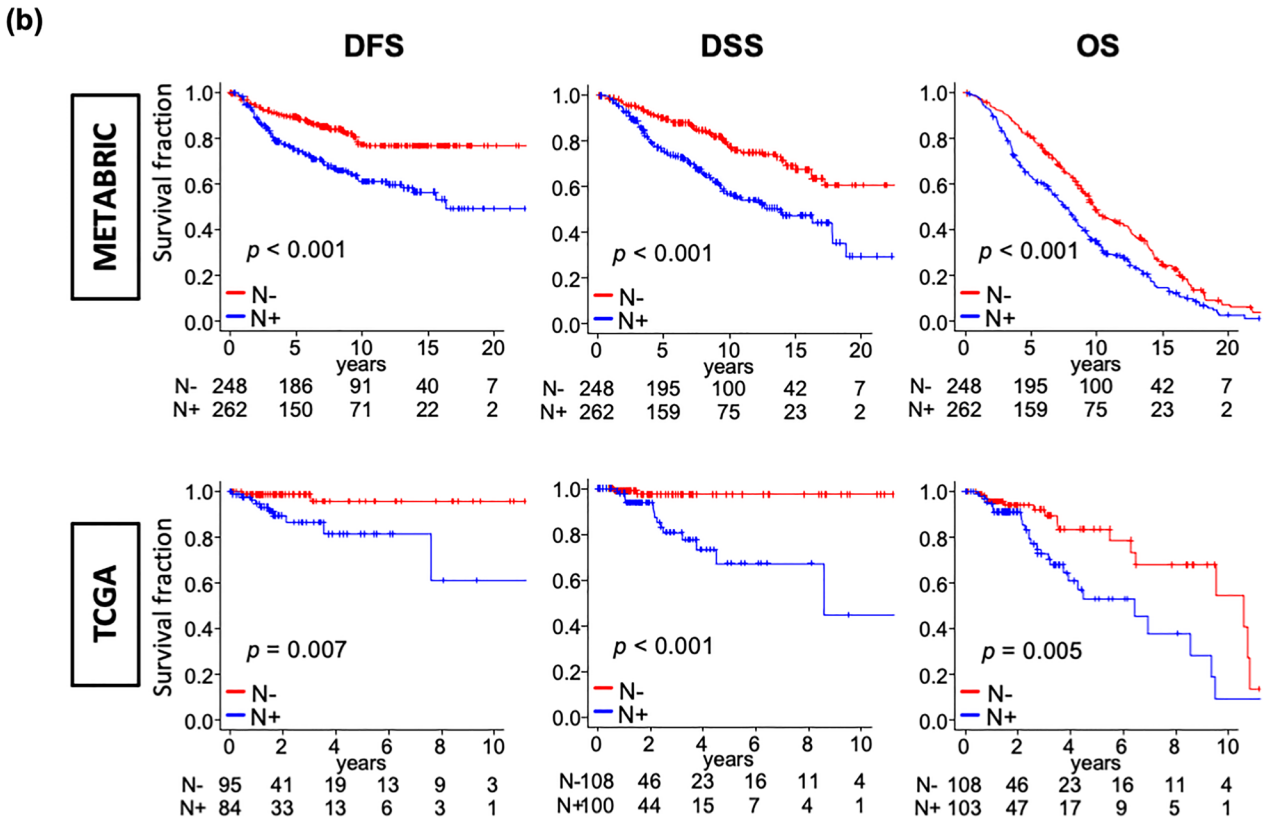
N+ 525 271 144 87 46 18
N- 506 286 158 97 53 21
N+ 540 283 153 92 49 18


(b)
DFS
DSS
OS
METABRIC
TCGA
Survival fraction
p < 0.001
p = 0.007
p = 0.005
N-
N+
years
N- 248 186 91 40 7
N+ 262 150 71 22 2
N- 248 195 100 42 7
N+ 262 159 75 23 2
N- 248 195 100 42 7
N+ 262 159 75 23 2
N- 95 41 19 13 9 3
N+ 84 33 13 6 3 1
N-108 46 23 16 11 4
N+100 44 15 7 4 1
N- 108 46 23 16 11 4
N+ 103 47 17 9 5 1

◂**Fig. 1** Oncologic survival outcomes of patients in the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas (TCGA) cohorts by axillary lymph node (LN) status involvement. Kaplan–Meier disease-free survival (DFS), disease-specific survival (DSS), and overall survival (OS) curves of the whole cohort (A) and older women (≥ 70 years old) in the METABRIC and TCGA cohorts. Comparison between LN-positive (blue line) and LN-negative (red line) patients was performed using log-rank test. The bottom one-third was used as cut-off value to divide two groups within cohorts

macrophages and high level of intratumor heterogeneity and fraction altered. Older women with breast cancers who had low expression level of *RUFY3* were more frequently diagnosed with LN-positive tumors, which translated into worse prognosis. Altogether low expression level of *RUFY3* may be associated with LN status in older women with breast cancer.

The role of *RUFY3* family of proteins in cancer has been previously studied by other groups. Xie et al. [38] reported that *RUFY3* levels were elevated in human colorectal cancer. Patients with a high expression of *RUFY3* had worse prognosis than those with a low expression. *RUFY3* was shown to play an important role in cancer progression and metastasis in both in vitro and in vivo experiments. Wang et al. [39] found that *RUFY3* overexpression promoted gastric cancer cell migration and invasion. Men et al. [40] reported that high *RUFY3* expression was associated with LN metastasis and advanced cancer stage. In patients with LN-positive tumors, expression rate of *RUFY3* (65.1%) was much higher than in those LN-negative tumors (43.5%). Although there was no statistically significant difference in the tumor size, there was a statistical difference in the expression of *RUFY3* across different cancer stages ($p < 0.01$). The authors speculated that *RUFY3* plays a role in LN metastasis rather than promoting tumor growth. Although the data presented in the current study support that intratumor *RUFY3* protein level is a marker of LN invasion as well as prognosis for breast cancer, these results differ in that low *RUFY3* expression levels were associated with these findings. Therefore, the role of *RUFY3* in breast cancer is not fully characterized with these data. In vitro and in vivo studies are necessary to understand the biological mechanism that explains these findings.

Data form other studies may be useful to hypothesize potential mechanisms behind the findings herein. In one study overexpression of *RUFY3* increased colorectal cancer cell proliferation and promoted colony formation in soft agar in vitro, whereas knockdown of *RUFY3* expression by siRNA inhibited colony formation in soft agar, migration, and invasion [41]. The authors concluded that *RUFY3* upregulation may be an important mechanism underlying

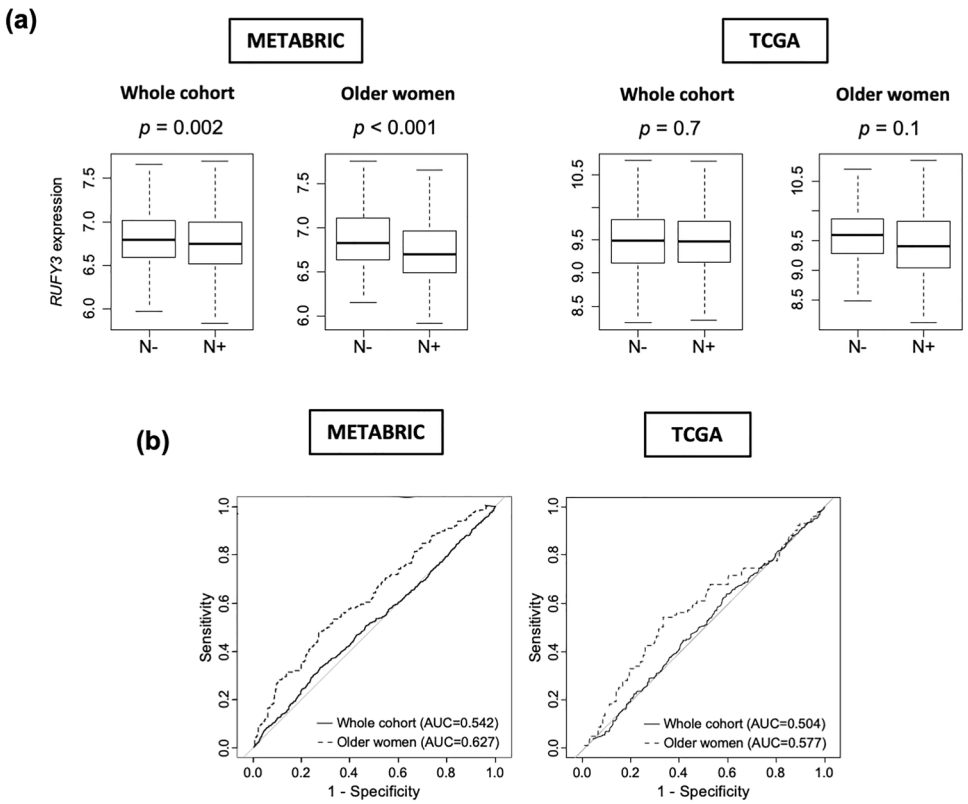


Fig. 2 *RUFY3* is associated with lymph node metastasis in patients with breast cancer. **a** Box plots depicting *RUFY3* expression stratified lymph node involvement in the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas (TCGA) cohorts. P-values were calculated by Mann–Whitney *U* test. **b** Receiver operator characteristic (ROC) curve showing the sensitivity and specificity of *RUFY3* among older women and all age women. Abbreviations: *N*− lymph node-negative; *N*+ lymph node-positive

Table 2 Characteristics of older women with breast cancer according to *RUFY3* expression levels in the METABRIC and TCGA cohorts

Variable	*RUFY3* low	*RUFY3* high	*p* value
METABRIC ($n=510$)	$n=168$	$n=342$	
Age (yo), median (IQR)	75 (73–80)	76 (73–79)	0.7
Molecular subtype			
ER+/HER2−	141 (83.9)	286 (83.6)	
TNBC	12 (7.1)	27 (7.9)	
HER2+	14 (8.3)	29 (8.5)	
Unknown	1 (0.6)	0 (0)	0.9
Nottingham grade			
1	12 (7.1)	32 (9.3)	0.6
2	79 (47.0)	158 (46.1)	
3	72 (42.8)	134 (39.1)	
Unknown	5 (2.9)	18 (5.2)	
AJCC stage			
0	0 (0)	1 (0.3)	< 0.001
I	1 (0.6)	59 (17.2)	
II	8148.2	155 (45.3)	
III	15 (8.9)	16 (4.6)	
IV	1 (0.6)	2 (0.5)	
Unknown	57 (33.9)	109 (31.9)	
TCGA ($n=211$)	$n=70$	$n=141$	
Age (yo), median (IQR)	75 (72–79)	77 (73–80)	0.07
Molecular subtype			
ER+/HER2−	36 (51.4)	88 (62.4)	0.1
TNBC	10 (14.2)	11 (7.8)	
HER2+	16 (22.8)	23 (16.3)	
Unknown	8 (11.4)	19 (13.4)	
T category			
T1	12 (17.1)	45 (31.9)	0.06
T2	44 (62.8)	65 (46.1)	
T3	9 (12.8)	24 (17.0)	
T4	5 (7.1)	7 (4.9)	
Unknown	0 (0)	0 (0)	
AJCC stage			
I	7 (10.0)	32 (22.7)	0.03
II	36 (51.4)	72 (61.0)	
III	26 (37.1)	32 (22.7)	
IV	1 (1.4)	1 (0.7)	
Unknown	0 (0)	4 (2.8)	

AJCC American Joint Committee on Cancer; *ER* estrogen receptor; *HER2* human epidermal growth factor 2; *IQR* interquartile range; *LN* lymph node; *METABRIC* Molecular Taxonomy of Breast Cancer International Consortium; *TCGA* The Cancer Genome Atlas; *TNBC* triple-negative breast cancer; *yo* years old

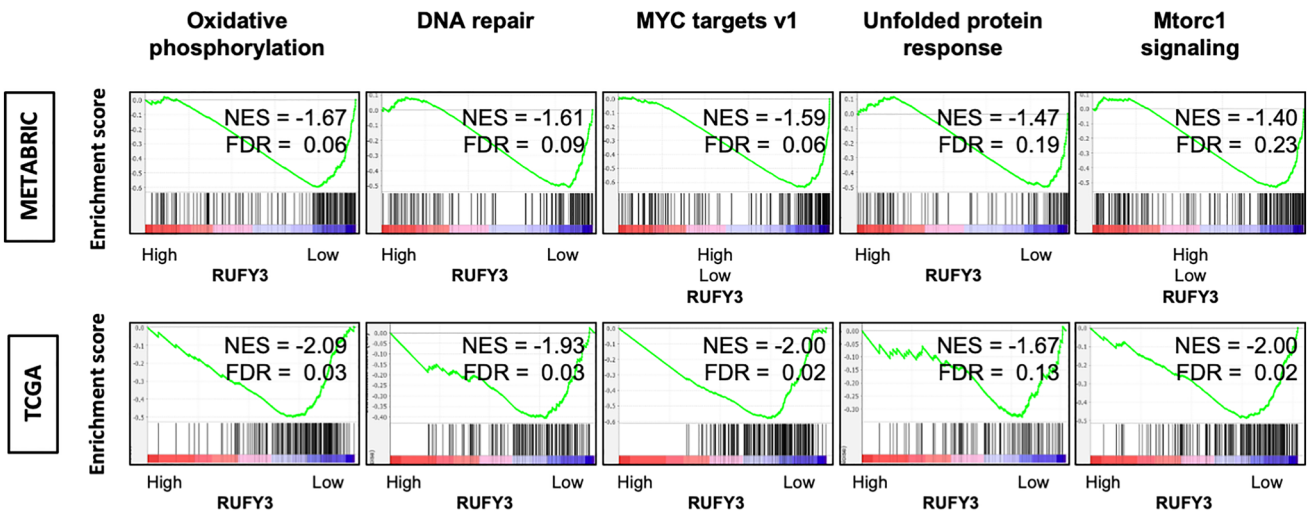


Fig. 3 Gene set enrichment analysis (GSEA) according to *RUFY3* expression level in older women with breast cancer in the METABRIC and TCGA cohorts. Enrichment plots with normalized enrichment score (NES) and false discovery rate (FDR) of hallmark gene sets which were significantly enriched in the low *RUFY3* groups in both cohorts. Bottom one-third was used as cut-off value to divide two groups within cohorts

tumor development in colorectal cancer cells. In this same study the authors noted that *RUFY3* interacted with *FOXK1* in colorectal cancer cells [41]. These results indicated that cancer progression and metastasis were promoted by a key signaling pathway involving a *RUFY3-FOXK1* axis. Another study suggested that *RUFY3* plays an important role in Ras-like GTPase signaling pathways [37]. In one study *RUFY3* overexpression led to the formation of F-actin-enriched protrusive structures at the cell periphery [39]. P21-activated kinase-1 (PAK1) interacts with *RUFY3*, resulting in RUFY3-induced gastric cancer cell migration [39]. Altogether these studies suggest that overexpression of *RUFY3* promotes cancer cell progression, migration, and metastasis. Interestingly, our study shows a unique role for *RUFY3* in breast cancer as low expression level is associated with more aggressive clinical features. Future in vitro and in vivo studies are necessary to study understand the how low expression levels of *RUFY3* produce negative clinical results.

Several other studies have shown that *RUFY3* is associated with poor prognosis [38, 40, 41]. In these studies, survival analyses showed that there was a negative association between *RUFY3* expression and OS across different types of cancers. Interestingly, Men et al. [40] showed through a single Cox regression that four clinical characteristics and *RUFY3* expression influenced patient prognosis. However, after adjusting for tumor size, LN metastasis, and TNM staging, multiple Cox regression revealed that *RUFY3* expression could be considered an independent factor for predicting survival in lung adenocarcinoma patients. On the other hand, *RUFY3* expression was not significantly associated with OS, whereas age, LN metastasis, and tumor size were in multivariable Cox regression analysis of the METABRIC breast cancer cohort (data not shown). This is most likely because *RUFY3* is a confounding factor of age and LN metastasis.

There are several limitations to this study. Although multiple large patient cohorts were used in this study to validate our findings, the data originated from retrospective studies from a publicly available source, which limit our access to clinical parameters. Furthermore, the cohorts represent a convenience sample of patients with tumors large enough to allow research tissue procurement who were treated at centers with robust tissue procurement programs. The cohorts' raw data vary in terms of patient demographics and clinical features; therefore, pooled and individual data analyses were performed to assess for heterogeneity. The cohorts also lack relevant clinical information, such as comorbidities, functional status, and treatment details. Additionally, data to explain the biological processes that support the findings in this study were not available given the bioinformatics approach.

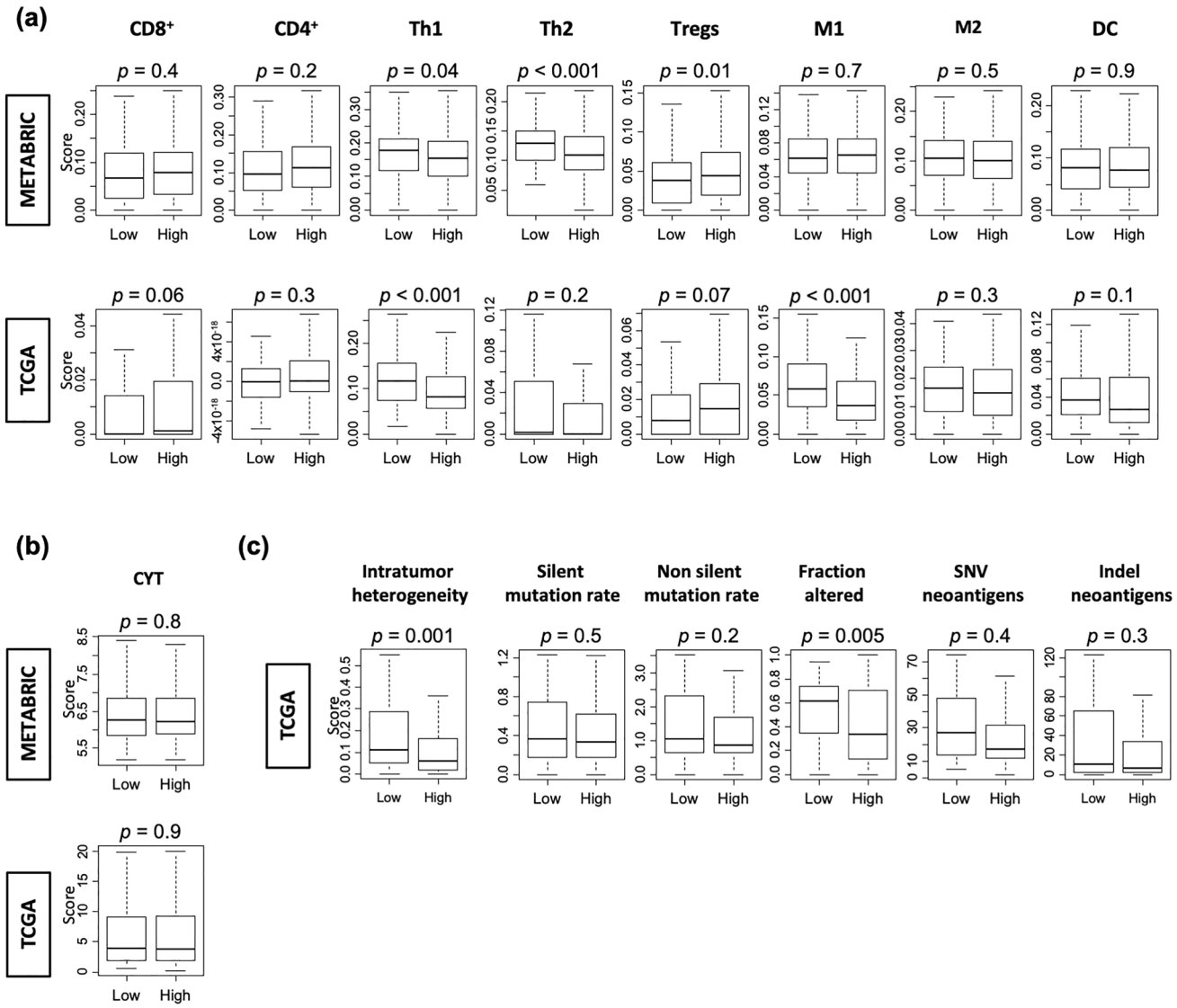


Fig. 4 Association of *RUFY3* expression level with immune fraction in the tumor microenvironment, immune activity, intratumor heterogeneity, and mutation load. Boxplots of **a** the infiltrating fraction of immune cells [$CD8^+$ T cells, $CD4^+$ T cells, T helper type1 (Th1) and type2 (Th2) cells, regulatory, M1 and M2 macrophages, and dendritic cells (DC)]; **b** cytolytic activity score (CYT); and **c** intratumor heterogeneity and mutation-related score [silent and non-silent mutation rate, fraction altered, single-nucleotide variant (SNV), and indel neoantigens] stratified by *RUFY3* expression level in older women with breast cancer

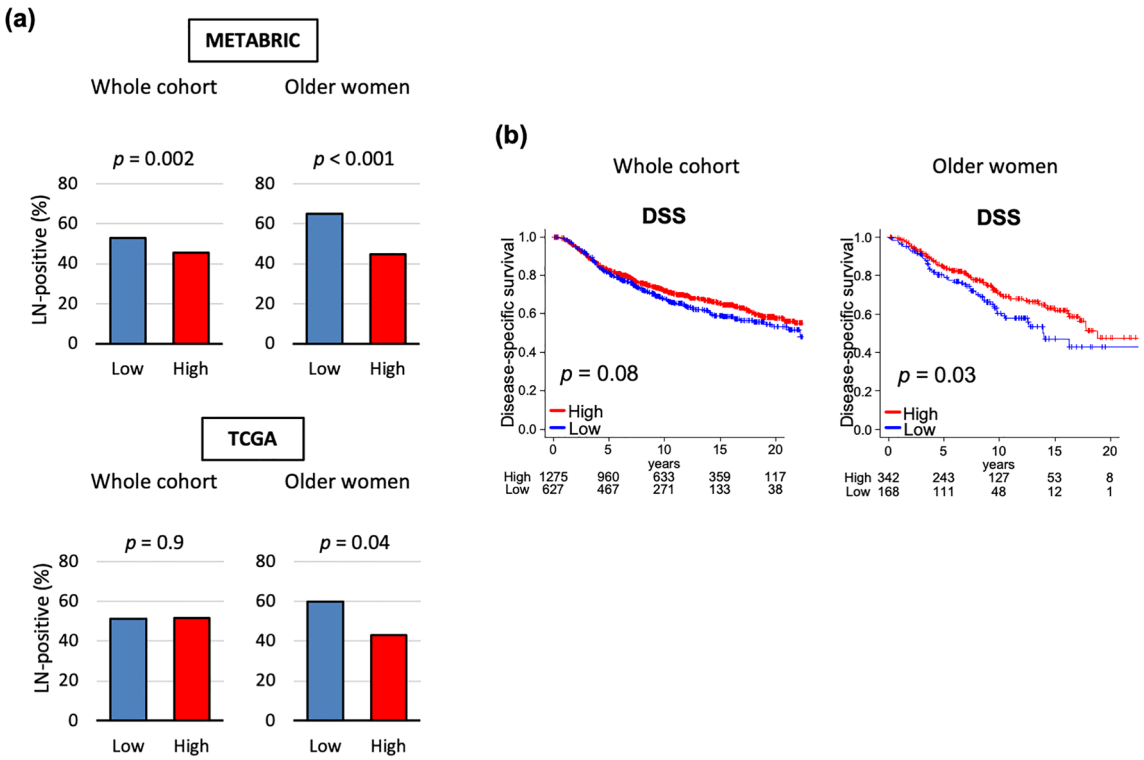


Fig. 5 Association of *RUFY3* expression level on LN involvement and patient survival. **a** Bar plots of lymph node metastasis rate by low and high *RUFY3* level in whole age cohort and older women in the METABRIC and TCGA cohorts. **b** Kaplan–Meier curves of disease-specific survival in whole age and older women cohorts of the METABRIC cohort

Conclusion

This study reports a novel role for *RUFY3* in breast cancer. Older women with breast cancers who had low expression level of *RUFY3* were more frequently diagnosed with LN-positive tumors, which translated into worse prognosis. Low expression level of *RUFY3* warrants further evaluation as an intratumor biomarker to predict LN metastasis in older women with invasive breast cancer.

Author contributions FAA, MO, and KT had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: FAA, MO, and KT. Acquisition, analysis, or interpretation of data: FAA, MO, and KT. Drafting of the manuscript: FAA, MO, SBE, and KT. Critical revision of the manuscript for important intellectual content: FAA, MO, AY, RM, SBE, and KT. Statistical analysis: FAA, MO, LY, and KT. Supervision on bioinformatic analyses: LY. Obtained funding: IE and KT. Administrative, technical, or material support: FAA, MO, RM, IE, and KT. Study supervision: KT.

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Data availability The datasets generated during and analyzed during the current study are available from the original source as they are publicly available deidentified databases.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Institutional review board (IRB) approval at Roswell Park Comprehensive Cancer Center (Buffalo, New York, United States of America) was waived as publicly available deidentified databases were used.

Consent to participate Not applicable.

Consent for publication Not applicable.

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