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# Expression of mTOR Signaling Pathway Molecules in Triple-Negative Breast Cancer

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## Keywords

Triple-negative breast cancer · mTOR · Glucose transporter

## Abstract

**Introduction:** Triple-negative breast cancer (TNBC), which lacks expression of estrogen receptor (ER), progesterone receptor (PgR), and epidermal growth factor receptor 2 (HER2), currently has no effective hormonal or molecular target therapy. **Objective and Methods:** To elucidate the role of the mammalian target of rapamycin (mTOR) signaling pathway in TNBC, the expression of molecules involved in mTOR signaling including mTOR, phosphorylated (p)-mTOR, p-4EBP1, GLUT1, GLUT3, HIF-1 $\alpha$ , and Ki67 was investigated by immunohistochemistry in 35 TNBC and 81 non-TNBC cases. **Results:** Expression of p-mTOR, the activated form of mTOR, but not unphosphorylated mTOR, was significantly higher in non-TNBC cases than in TNBC cases. Expression of p-4EBP1, GLUT1, and GLUT3 was higher in TNBC cases than in non-TNBC cases. When the localization of p-mTOR was classified as nuclear, perinuclear, or cytoplasmic, nuclear localization of p-mTOR was observed more frequently in TNBC than in non-TNBC cases and was correlated with the expression of GLUT1 and GLUT3, which was related

to proliferation activity examined with Ki67. **Conclusions:** mTOR signaling regulates cell proliferation in some cases of TNBC and may be a potential target of molecular therapy for TNBC.

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## Introduction

Triple-negative breast cancer (TNBC) is defined as a tumor without expression of estrogen receptor (ER), progesterone receptor (PgR), or epidermal growth factor receptor 2 (HER2). Because the differentiation and proliferation of TNBC cancer cells are independent of hormonal regulation and the HER2 signaling pathway, no effective therapy has been established to treat this subgroup of breast cancer patients [1]. Alternative signaling pathways governing tumor cell biology will have to be elucidated to identify new therapeutic targets and biomarkers for the early detection of TNBC tumors. Here, we investigated the expression of mammalian target of rapamycin (mTOR) signaling pathway (one of the cell growth pathways involved in the uptake of sugar and amino acids) molecules in TNBC.

mTOR is a serine/threonine kinase that controls transcription, translation, and autophagy to positively regulate growth factors, metabolism, and survival [2]. Although several drugs that inhibit mTOR have undergone clinical trials for the treatment of breast cancer [3], including TNBC [4], targeted therapies for TNBC remain unavailable [5]. mTOR is a component of the complex known as mTORC1, which also includes mLST8 (GβL) and Raptor (regulatory-associated-protein of mTOR) and promotes protein translation through a variety of interactions. One such mechanism is phosphorylation of the key translational regulators p70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) by phosphorylated (p)-mTOR, the activated form of mTOR. Under basal conditions, S6K is bound to the eukaryotic initiation factor 3 (eIF3) translation initiation complex. mTORC1 activity promotes the phosphorylation of S6K, which results in its dissociation from eIF3 [6] and enhances the translation of mRNAs bearing 5'-terminal oligopyrimidine tracts, such as those encoding ribosomal proteins [7, 8]. Phosphorylation of 4EBP1 (p-4EBP1) increases mRNA translation by inhibiting the interaction between 4EBP1 and eukaryotic initiation factor 4 (eIF4), thereby releasing a block to the formation of the translation initiation complex [9]. mTOR also acts as a positive regulator of hypoxia inducible factor (HIF-1), a transcription factor that regulates many genes involved in tumor-induced angiogenesis [10]. HIF-1 enhances glycolytic metabolism by promoting the expression of glucose transporters [11]. It has been reported that HIF-1 and glucose transporter 1 (GLUT1) protein expression levels are diminished with rapamycin treatment [12]. The expression of GLUT3 has also been shown to depend on mTOR activity, and to require the transcription factor HIF-1α [13].

In this study, we have compared the expression of mTOR signaling pathway molecules in TNBC and non-TNBC, with the aim of identifying potential therapeutic and diagnostic markers targeted specifically to TNBC.

## Materials and Methods

### Patient Selection

One hundred and sixteen invasive ductal carcinomas surgically resected at Toho University Medical Center Omori Hospital from 2008 to 2010 were collected. Cases of lobular carcinoma and other special histological types were omitted. Cases receiving preoperative chemotherapy and radiation therapy were also omitted. The cases were routinely examined for the expression of ER, PgR, and HER2, using the following primary antibodies and staining kit: anti-ER (Nichirei, Tokyo, Japan), anti-PgR (Thermo Scientific,

**Table 1.** Clinicopathological features of breast cancer in this study

	TNBC (n = 35)	Non-TNBC (n = 81)	p
Age, years	62±14	61±14	ns
Males	0	0	ns
Females	35	81	ns
Tumor size, mm	32±20	20±12	<0.01 <sup>b</sup>
Lymph node status			ns
Positive for metastasis	14	15	
Negative for metastasis	19	57	
Hormone receptor-positive (ER or PgR)	0 (0%)	72 (89%)	
HER2-positive	0 (0%)	14 (17%)	
Nuclear grade [21]			<0.01 <sup>a</sup>
1	1	36	
2	10	24	
3	24	21	
Histological grade			<0.01 <sup>a</sup>
1	1	22	
2	13	44	
3	21	15	

Values express *n* or mean ± SD. ns, not significant; TNBC, triple-negative breast cancer; ER, estrogen receptor; PgR, progesterone receptor.

<sup>a</sup> Statistically significant,  $\chi^2$  test; <sup>b</sup> statistically significant, Mann-Whitney U test.

Waltham, MA, USA), and HercepTest (Dako, Carpinteria, CA, USA). For ER and PgR, cases were considered positive when >1% of cancer cells showed positive immunoreactivity. For HercepTest, cases were considered negative if they scored 0 or +1, and positive if they scored +3. Weakly positive cases (2+) were considered equivocal and were further evaluated by *HER2* gene amplification using the FISH method. In this study, FISH was performed for 2 cases, neither of which showed *HER2* gene amplification. When all 3 markers were negative, the cases were considered to be TNBC. By these criteria, a total of 35 cases were classified as TNBC and 81 as non-TNBC. The clinicopathological characteristics of the TNBC and non-TNBC tumors are summarized in Table 1. Prognostic information was collected from medical charts. The mean follow-up time was 71 (range 1–123) months.

### Immunohistochemistry

We examined formalin-fixed and paraffin-embedded 4- $\mu$ m sections from 1 representative paraffin block per case for mTOR, p-mTOR, p-4EBP1, GLUT1, GLUT3, HIF-1 $\alpha$ , and Ki67. The sources of the antibodies for these antigens are summarized in Table 2. Endogenous peroxidase was blocked with 0.5% hydrogen peroxide in methanol for 30 min. Before the primary antibody was applied, slides were subjected to antigen retrieval (Table 2). After application of the primary antibodies, the slides were washed with PBS and then incubated with secondary EnVision+System-HRP-labeled polymer (Dako). Finally, the tissue sections were visualized with Stable DAB (Falma, Tokyo, Japan) and counterstained with Mayer's hematoxylin.

**Table 2.** Antibodies used and their conditions of immunostaining

Antigen	Antibody	Dilution	Antigen retrieval (buffer)	Supplier
mTOR	rabbit, monoclonal	×50	microwave for 20 min (pH6) <sup>a</sup>	Cell Signaling Technology, Danvers, MA, USA
phospho-mTOR (Ser2448)	rabbit, monoclonal	×100	microwave for 20 min (pH6) <sup>a</sup>	Cell Signaling Technology
phospho-4E-BP1 (Thr37/46)	rabbit, monoclonal	×1,000	autoclave for 10 min (pH6) <sup>b</sup>	Cell Signaling Technology
GLUT1	rabbit, polyclonal	×200	microwave for 10 min (pH6) <sup>a</sup>	Abcam, Cambridge, UK
GLUT3	rabbit, polyclonal	×200	microwave for 10 min (pH6) <sup>a</sup>	Abcam
HIF-1α	mouse, polyclonal	×100	autoclave for 10 min (pH9) <sup>b</sup>	BD Transduction Laboratories, San José, CA, USA
Ki67	rabbit, polyclonal	×100	autoclave for 10 min (pH6) <sup>b</sup>	Thermo Scientific, Waltham, MA, USA

<sup>a</sup> Microwave of 500 W was used for antigen retrieval; <sup>b</sup> autoclave for antigen retrieval was performed at 120°C.

Both the intensity and the area of immunohistochemical staining in the cancer tissue sections were evaluated, and the immunoreactivity score was calculated. The proportion of areas exhibiting positive staining was scored as follows: 1 (focal), 1–10%; 2 (partial), 11–30%; and 3 (diffuse), >30%. The staining intensity score, ranging from 0 to 3, was evaluated as follows: 0, no staining; 1, weak; 2, moderate; and 3, strong. The multiplication of the proportion score and the intensity score was taken as the immunoreactivity score.

Subsequently, the cellular localization of p-mTOR was examined, and the cases were classified into 3 categories: nuclear, perinuclear, and cytoplasmic expression. Ki67 expression was also evaluated in >500 cancer cells, and the proportion of Ki67-positive cells (the number of Ki67 positive cells/number of total cancer cells) was calculated as a percentage.

#### Statistical Analysis

Spearman's rank correlation test, the Mann-Whitney U test, and the  $\chi^2$  test with R software v3.2.2 were used to assess statistical significance. Prognosis was analyzed by the Kaplan-Meier method along with the log rank test.  $p < 0.05$  was considered statistically significant.

## Results

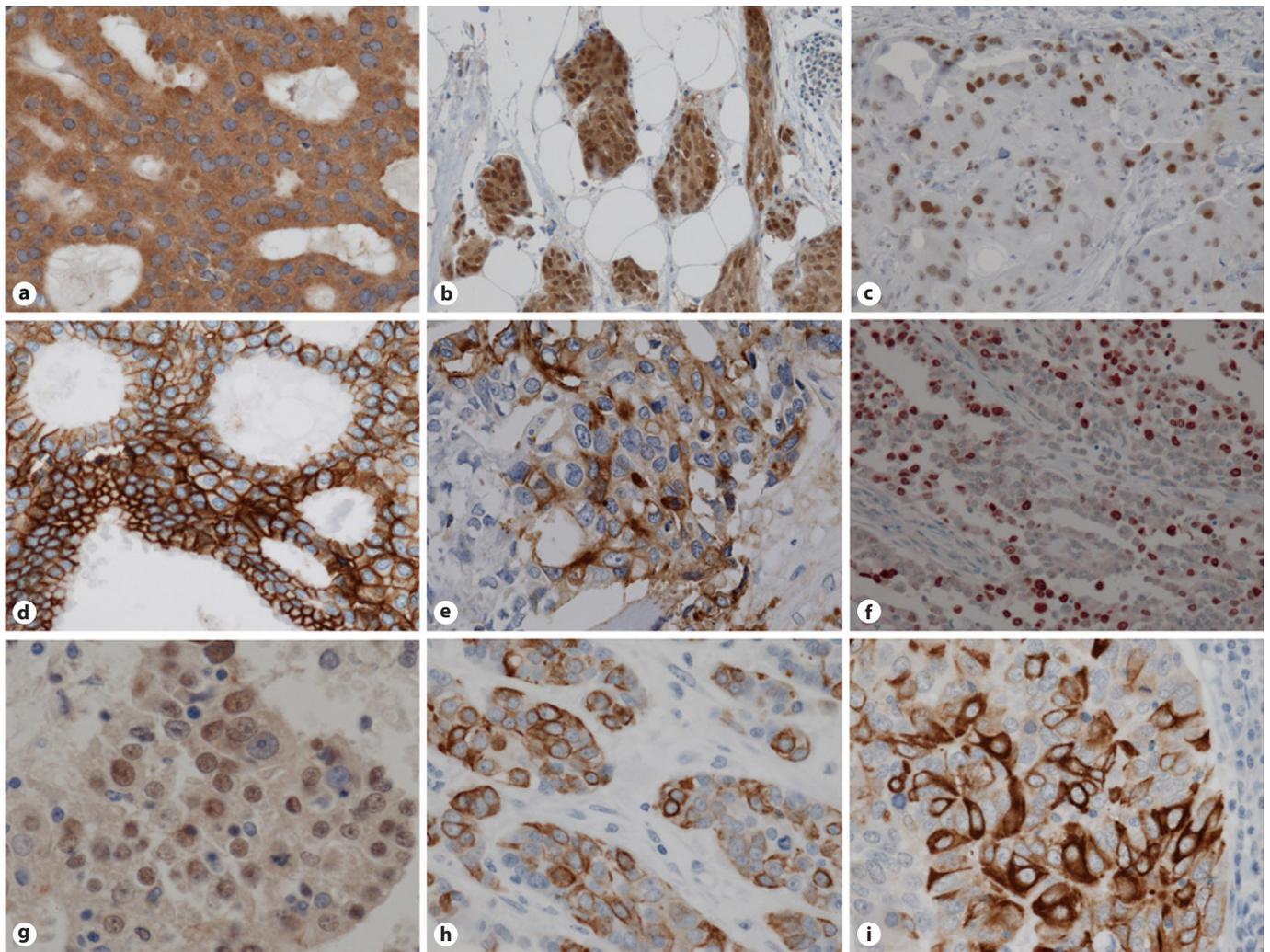
### Expression of Each Molecule

In normal mammary glands, mTOR showed weak cytoplasmic expression in epithelial cells in mammary ducts and lobules. On the other hand, the p-mTOR level varied; it was detected in the cytoplasm of epithelial cells in some cases but not others. The expression of Nuclear p-4EBP1 and membranous GLUT3 was low or negative. There was no GLUT1 expression in the epithelial cells of normal mammary glands, but high expression in myoepithelial cells was observed in a few cases.

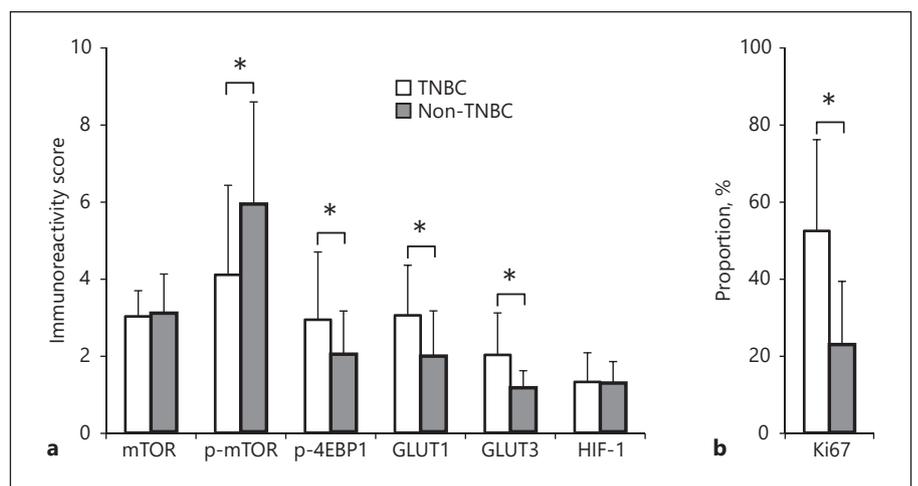
In both the TNBC and non-TNBC cases, expression of mTOR was observed in the cytoplasm, GLUT1 and

GLUT3 at the cell membrane, and p-4EBP1 and HIF-1α in the nucleus, and p-mTOR in both the cytoplasm and nucleus (Fig. 1). p-mTOR expression was first evaluated in the whole cell without considering subcellular localization. Expression of p-mTOR was significantly lower in TNBC than in non-TNBC, but mTOR expression was not significantly different between the tumor types. In contrast to p-mTOR, the expression of p-4EBP1, GLUT1, and GLUT3 was significantly higher in TNBC than in non-TNBC cases (Fig. 2). A positive weak correlation of expression was observed between GLUT1 and p-4EBP1 ( $\rho = 0.246$ ,  $p = 0.0079$ ), and between GLUT3 and p-4EBP1 ( $\rho = 0.267$ ,  $p = 0.0037$ ). In addition, the expression of GLUT1 was positively correlated with that of HIF-1α ( $\rho = 0.336$ ,  $p = 0.0002$ ) and GLUT3 ( $\rho = 0.461$ ,  $p < 0.0001$ ) (Table 3).

To identify whether the expression of mTOR, p-mTOR, p-4EBP1, GLUT1, GLUT3, and HIF-1α was related to prognostic factors, the correlation with clinicopathological factors such as the age of the patient, tumor size, nuclear grade, histological grade, lymph node status, hormone receptor status, and HER2 overexpression was tested. However, no significant relationship between the expression of these proteins and the clinicopathological factors was observed. There was also no significant correlation with patient prognosis (overall survival). Specifically, overall survival was compared in patients with high and low expression of p-mTOR among 116 cases, but no significant difference was found (online suppl. Fig. 1; for all online suppl. material, see [www.karger.com/doi/10.1159/000503311](http://www.karger.com/doi/10.1159/000503311)). Among the TNBC cases, no significant difference in prognosis was observed between the cases with nuclear p-mTOR expression and those with perinuclear or cytoplasmic expression (data not shown).



**Fig. 1.** **a–e** Immunohistochemical staining of mTOR signaling pathway molecules. **a** mTOR. **b** p-4EBP1. **c** HIF-1. **d** GLUT1. **e** GLUT3. **f** Immunohistochemical staining of the Ki67 antigen. **g–i** Localization of p-mTOR, as detected by immunohistochemistry, in the nucleus (**g**), perinucleus (**h**), and cytoplasm (**i**).



**Fig. 2.** Expression of each marker in triple-negative and non-triple-negative breast cancer. TNBC, triple-negative breast cancer. \*  $p < 0.05$ , statistically significant.

**Table 3.** Spearman's rank correlation among the expression of the molecules

	$\rho$	$p$ value
mTOR vs. GLUT1	0.155	0.0961
mTOR vs. GLUT3	-0.0331	0.724
mTOR vs. p-4EBP1	-0.0081	0.931
p-mTOR vs. GLUT1	-0.0736	0.432
p-mTOR vs. GLUT3	-0.156	0.0951
p-mTOR vs. p-4EBP1	0.111	0.237
GLUT1 vs. GLUT3	0.461	<0.0001
GLUT1 vs. p-4EBP1	0.246	0.0079
GLUT1 vs. HIF-1	0.336	0.0002
GLUT3 vs. p-4EBP1	0.267	0.0037
Ki67 vs. mTOR	-0.0001	0.998
Ki67 vs. p-mTOR	-0.321	0.0004
Ki67 vs. GLUT1	0.496	<0.0001
Ki67 vs. GLUT3	0.579	<0.0001
Ki67 vs. p-4EBP1	0.192	0.0395
Ki67 vs. HIF-1	0.201	0.03

Proportion of cells positive for Ki67. Immunoreactivity score for mTOR, p-mTOR, p-4EBP1, HIF-1, GLUT1, and GLUT3.

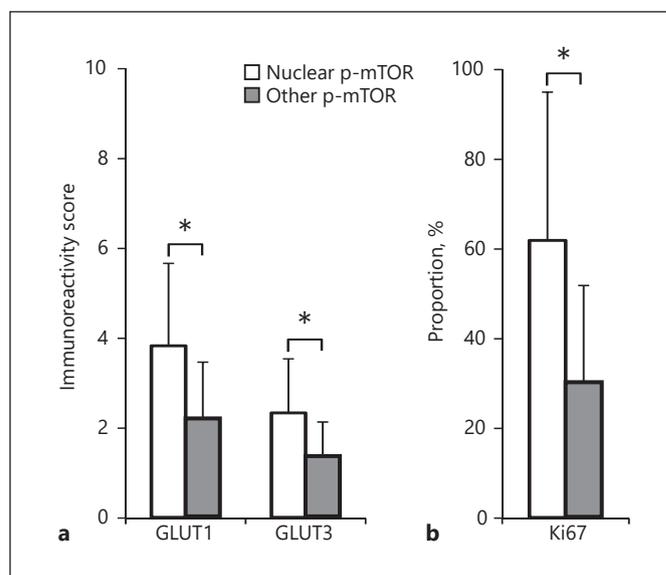
**Table 4.** Localization of p-mTOR in cases of TNBC and non-TNBC

	$n$	Nuclear	Perinuclear	Cytoplasmic
TNBC	35	5 (14%)	6 (17%)	24 (68%)
Non-TNBC	81	1 (1%)	19 (23%)	61 (75%)
Total	116	6 (5%)	25 (22%)	85 (73%)
$p$ value		0.009	0.623	0.49

Values express  $n$  (%). TNBC, triple-negative breast cancer.

#### Localization of mTOR and p-mTOR

In all TNBC and non-TNBC cases, both mTOR and p-mTOR were assigned an immunoreactivity score of at least 1. mTOR expression was confined to the cytoplasm. However, among the 35 TNBC cases, nuclear p-mTOR was expressed in 5 (14%), perinuclear p-mTOR in 6 (17%), and cytoplasmic p-mTOR in 24 (68%). On the other hand, in the 81 non-TNBC cases, nuclear p-mTOR, perinuclear p-mTOR, and cytoplasmic p-mTOR were found in 1 (1%), 19 (23%), and 61 (75%) cases, respectively (Fig. 1) (Table 4). Expression of GLUT1 and GLUT3 was found to be significantly higher in cases with nuclear p-mTOR expression than in those with perinuclear or cytoplasmic expression (Fig. 3a, 4).



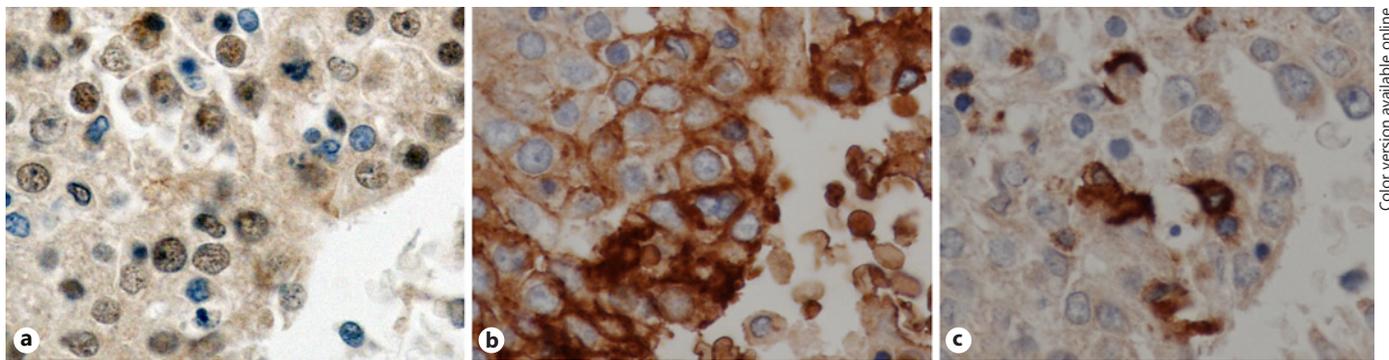
**Fig. 3.** Immunoreactivity scores and Ki67 proportion according to p-mTOR localization. \*  $p < 0.05$ , statistically significant.

#### Relation with Proliferation Activity

The proportion of Ki67-positive cells was significantly higher in TNBC cases than in non-TNBC cases (mean  $\pm$  SD:  $52 \pm 24$  vs.  $23 \pm 16\%$ ;  $p < 0.0001$ ) (Fig. 2b). The proportion of Ki67-positive cells correlated with the immunoreactivity scores for GLUT1 and GLUT3, but not with those for mTOR and p-mTOR (Table 3). In addition, the proportion of Ki67-positive cells was higher in the cases with nuclear p-mTOR expression than in those with perinuclear or cytoplasmic expression (Fig. 3b).

#### Discussion

Therapeutic inhibition of the PI3K/mTOR pathway in breast cancer is an active area of research. Several ongoing studies are evaluating the effect of PI3K/mTOR inhibitors in TNBC, administered alone or in combination with chemotherapy [4]. Most previous studies that evaluated p-mTOR expression in breast cancer did not consider the breast cancer subtype, with the exception of 3 studies that did describe p-mTOR expression in TNBC [14–16]. Ueng et al. [15] reported p-mTOR positivity in 72% of TNBC tumors from 177 patients. Bakarakos et al. [16] described a positive association between p-mTOR expression and lymph node status and a negative impact of p-mTOR expression on overall survival. We evaluated the immunohistochemical expression of p-mTOR in 116 breast can-



Color version available online

**Fig. 4.** Immunohistochemical staining of p-mTOR, GLUT1, and GLUT3 using sequential tissue sections from a case of TNBC. **a** p-mTOR nuclear expression. **b** Diffuse strong expression of GLUT1. **c** Strong expression of GLUT3 in a portion of cancer cells.

cer specimens and found that 63 (54%) had an immunoreactivity score >6. This suggests that >50% of breast cancer patients are potential candidates for treatment with p-mTOR inhibitors. Our findings are corroborated by Walsh et al. [14], who detected increased p-mTOR expression in TNBC versus non-TNBC.

Our results regarding the relationship between p-mTOR and GLUT expression appear contradictory when taking the mTOR signaling pathway into consideration. In the mTOR signaling pathway, increased expression of p-mTOR causes increased expression of p-4EBP1 via HIF-1 $\alpha$ , leading to increased GLUT expression [2, 12]. On the other hand, in our data, although p-mTOR increased in non-TNBC versus TNBC, the p-4EBP1 expression was higher in TNBC than in non-TNBC. HIF-1 $\alpha$  expression is controlled by mTOR in several ways and under different conditions, including hypoxia and mitogenic stimulation [17].

Other studies have suggested an HIF-1 $\alpha$ -dependent regulation of GLUT1 [12] and GLUT3 [13]. We therefore examined the intracellular localization of p-mTOR. While mTOR localizes predominantly in the cytoplasm under steady-state conditions, cytoplasmic/nuclear shuttling of mTOR regulates 4EBP1 phosphorylation [18]. In this study, comparing the localization of p-mTOR between TNBC and non-TNBC cases, p-mTOR nuclear expression was more frequently observed in TNBC, which is consistent with a previous report by Walsh et al. [14]. In addition, the expression of p-mTOR in the nucleus correlated with that of GLUT1 and GLUT3. Our data indicate that p-mTOR in the nucleus may play an important role, particularly in TNBC.

Vazquez-Martin et al. [19] observed that nuclear enrichment of p-mTOR is closely related to the proliferative capacity of breast cancer cell lines. In our study, the ex-

pression of Ki67 (reflecting proliferative activity) was positively correlated with that of GLUT1 ( $\rho = 0.496$ ) and GLUT3 ( $\rho = 0.579$ ). Ki67 index is an important factor when considering prognosis [20]. However, in this study, no direct relationship between prognosis and GLUT1 or GLUT3 expression could be demonstrated.

Although treatment of breast cancer with mTOR inhibitors has been reported [3, 4], it may not be an effective therapeutic strategy at present. Furthermore, mTOR localization was not examined in previous clinical trials of mTOR inhibitors [3–5]. Our results may be helpful for identifying those patients most likely to benefit from mTOR inhibition by examining p-mTOR localization.

#### Statement of Ethics

The study protocol was approved by the Ethics Committee of Toho University School of Medicine (27119).

#### Disclosure Statement

There were no disclosures.

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