Phosphorylated S6 Kinase-1: A Breast Cancer Marker Predicting Resistance to Neoadjuvant Chemotherapy

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Abstract. *Background: Pre-clinical data support a link between the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway and chemoresponsiveness. We evaluated whether the expression of phosphorylated AKT (p-AKT) or phosphorylated S6 kinase-1 (p-S6K1), a key effector of the mTOR pathway, could be a predictive marker for chemoresponsiveness in breast cancer. Patients and Methods: A total of 209 patients with locally advanced breast cancer who received neoadjuvant chemotherapy between April 2005 and July 2012 were analyzed. Patients without a minimum of 10% tumor reduction, after neoadjuvent chemotherapy, were classified as non-responders. Results: Overall, 184 (88%) patients were classified as responders and 25 (12%) as non-responders. The positive expression rate for p-AKT and p-S6K1 was 31.6% and 45%, respectively. There was no difference in the pre-chemotherapy clinical stage according to p-S6K1 or p-AKT expression status. p-AKT expression was slightly higher in non-responders compared to responders (48% vs. 30.9%; p=0.088). However, p-S6K1 expression was significantly higher in non-responders than responders (68% vs. 41.8%; p=0014). Following multivariate analysis, p-S6K1 positivity remained an independent predictor of non-responder status (hazard ratio=3.81; 95% confidence interval=1.28-11.31; p=0.016). Conclusion: The expression of p-S6K1 may be a predictive marker of resistance to neoadjuvant chemotherapy in patients with breast cancer.*

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Key Words: Breast cancer, neoadjuvant chemotherapy, chemo-responsiveness, S6 kinase 1 (S6K1), AKT.

Neoadjuvant chemotherapy (NAC) was first described for patients with locally advanced breast cancer (LABC) in the late 1970s (1) and was initially used to convert patients with inoperable LABC to surgical candidates. However, this type of chemotherapy has become more common for patients with operable disease in order to down-tage the tumor and enable breast-conserving surgery (BCS) in those who may have otherwise required a mastectomy (2-5). Additionally, NAC allows for *in vivo* assessment of tumor response to systemic therapy, unlike in the adjuvant setting. Importantly, clinical trials have shown that patients who achieved a pathological complete response (pCR) after NAC had improved survival compared with those who did not achieve pCR (5).

Systemic chemotherapy is one of the most crucial factors for reducing mortality in women with breast cancer. However, chemotherapy resistance remains the main problem for patients with cancer (6). Numerous patients do not respond positively to chemotherapeutic agents and instead, suffer from its adverse effects. However, the mechanisms of resistance are not well-understood, and no clinically useful predictive markers of a patient's response to chemotherapy have been defined. Molecular predictors that could aid the identification of patients who may benefit from systemic chemotherapy would be an important step towards personalized medicine.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway regulates essential cellular functions, including cell survival, proliferation, metabolism, migration, and angiogenesis (7-9). There is growing evidence that the PI3K/AKT/mTOR pathway is frequently de-regulated during tumorigenesis, *via* genetic and epigenetic alterations, contributing to the development and progression of human cancer (10). Ribosomal S6 kinase-1 (S6K1) and the eukaryotic translation initiation factor 4E-binding protein (4E-BP1) are the two main downstream effectors of mTOR (11). The S6K1 gene, *RPS6KB1*, localized to the chromosomal region 17q23, is amplified in several breast cancer cell lines and

approximately 30% of primary tumors (12-14). *RPS6KB1* amplification or protein expression has been linked to poor prognosis in patients with breast cancer, supporting its role in disease development and progression (12, 15, 16).

Pre-clinical data support a link between the PI3K/AKT/mTOR signaling pathway and chemoresponsiveness. In ovarian cancer cells, taxanes interact with this pathway to promote cell death and AKT activation promotes taxane resistance (17, 18). In prostate cancer cells, a high expression of phosphorylated AKT (p-AKT) and phosphorylated S6K1 (p-S6K1) were associated with doxorubicin resistance (19). In the present study, we investigated whether the expression of p-AKT or p-S6K1 could be a predictive marker for chemoresponsiveness by assessing 209 patients with LABC who received NAC.

Patients and Methods

Study patients and treatment. The Korea Cancer Center Hospital Breast Cancer Center (KCCHBCC) database is a prospectively maintained, web-based database and has been described elsewhere (20, 21). From the KCCHBCC database, patients who underwent NAC and subsequent breast surgery between April 2005 and July 2012 were identified. Patients with metastatic or recurrent breast cancer, those who received non-anthracycline and taxane-based chemotherapy or trastuzumab-containing regimens, and those who received NAC at other institutions were excluded. A total of 209 patients were included in the analysis. Clinicopathological information such as age, treatment regimen, clinical and pathological TNM stage, histological grade, and molecular phenotype, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) status, were extracted from the database. The study protocol was reviewed and approved by the Institutional Review Board at the Korea Institute of Radiological and Medical Sciences (K-1307-002-009). The recommendations of the Declaration of Helsinki for biomedical research involving human subjects were also followed.

The NAC regimen comprised of docetaxel (75 or 60 mg/m^2) plus doxorubicin (60 or 50 mg/m^2) or epirubicin (75 or 60 mg/m^2) *via* intravenous infusion every three weeks for 3-6 cycles. Few patients received four cycles of doxorubicin (60 or 50 mg/m^2) and cyclophosphamide (600 mg/m^2) followed by four cycles of paclitaxel (175 or 140 mg/m^2) or docetaxel (75 or 60 mg/m^2) as NAC. After NAC completion, the patients underwent primary surgery and received three or more cycles of adjuvant chemotherapy, followed by radiation or hormonal therapy, if indicated.

Immunohistochemical analysis and assessment of response to NAC. Formalin-fixed, paraffin-embedded tumor tissue blocks were used for immunohistochemistry. The tissue sections were immunohistochemically stained with the appropriate antibodies for ER, PR, and HER2. Positive ER or PR staining was defined as staining of ≥10% nuclei in 10 high-power fields, and HER2 positivity was defined as 3(+) on immunohistochemical staining or *HER2* gene amplification by fluorescence *in situ* hybridization (FISH) or silver *in situ* hybridization (SISH).

The p-AKT and p-S6K1 expression status of the primary tumors was assessed by immunohistochemistry with a mouse monoclonal antibody against p-AKT (Cell Signaling Technology, Inc., Danvers, MA, USA; dilution 1:200) and p-S6K1 (Cell Signaling Technology, Inc.; dilution 1:50), respectively. Interpretation of p-AKT and p-S6K1 immunohistochemical staining has been previously described in detail (16, 21). Briefly, the immunoreactivities of p-AKT and p-S6K1 were interpreted in a semi-quantitative manner using an intensity-proportion scoring system, and the score was calculated by the sum of the intensity and proportion scores; this provided a score between 0 and 6. The proportion score was as follows: 0, no positive cells; +1, fewer than one-third positive tumor cells; +2, one-third to two-thirds positive tumor cells; and +3, more than two-thirds positive tumor cells. The intensity score was as follows: +1, weak staining; +2, intermediate staining; and +3, strong staining. A score of 0 was regarded as negative, while the other scores were regarded as positive for the statistical analysis.

Primary tumor size before NAC (D0) was measured by ultrasonography, chest computed tomography (CT), or contrast-enhanced magnetic resonance imaging (MRI), and the post-NAC tumor size (D1) was evaluated by pathological tumor size after surgery. The tumor reduction rate was calculated as follows: (D0–D1)/D0×100 (%). pCR was defined as the absence of residual invasive tumor cells in the breast (5).

Statistical analysis. The Pearson's chi-square test and Student's *t*-test were used to compare nominal and continuous variables between groups, respectively. In multivariate analysis, binary logistic regression analysis was performed to identify the factors predicting resistance to NAC. All statistical analyses were performed using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL., USA), and a p-value <0.05 was considered statistically significant.

Results

Clinicopathological characteristics. A total of 209 patients with a mean age of 49.97 years (±9.51 years) were investigated. The clinicopathological characteristics are presented in Table I. Most of the patients (90.4%) had infiltrating ductal carcinoma, and 26 patients (12.4%) had inflammatory breast cancer. One hundred and five (50.2%) cases were ER-positive, 115 (55%) were PR-positive, and 83 (39.7%) were HER2-positive. Using the criteria described above, the positive expression rate for p-AKT and p-S6K1 was 31.6% and 45%, respectively.

Most of the patients (97.1%) received docetaxel plus doxorubicin or epirubicin as a NAC regimen. The mean pre-NAC tumor size was 4.74 cm (±2.5 cm), and mean pathological tumor size after surgery was 2.41 cm (±2.3 cm). The mean tumor reduction rate after NAC was 48.6% (±37.8%). One hundred and eighty-four patients (88%) responded to NAC with a minimum 10% reduction in tumor size, including 15 cases of pCR (7.2%). On the contrary, 25 patients (12%) had a lower response, resulting in <10% tumor size reduction.

The clinicopathological characteristics according to p-S6K1 and p-AKT expression are compared in Table II. There were no significant differences in the variables according to p-AKT or p-S6K1 expression status, but the PR-positive rate was higher in both the p-AKT-positive and p-S6K1-positive

Table I. *Clinicopathological details of the study population.*

Variable		No. of patients (%)
Age, years	Mean (±sd)	49.97 (±9.51)
	<50	98 (46.9)
	≥50	111 (53.1)
Histopathological type	IDC	189 (90.4)
	Other	20 (9.6)
Grade	1/2	81 (57.9)
	3	59 (42.1)
	Unknown	69
Inflammatory breast cancer	Yes	26 (12.4)
	No	183 (87.6)
ER	Negative	104 (49.8)
	Positive	105 (50.2)
PR	Negative	94 (45.0)
	Positive	115 (55.0)
HER2	Negative	126 (60.3)
	Positive	83 (39.7)
p-AKT	Negative	134 (64.1)
	Positive	66 (31.6)
	Unknown	9 (4.3)
p-S6K1	Negative	115 (55.0)
	Positive	94 (45.0)
NAC regimen	D/A or D/E	203 (97.1)
	Other (A/C-D or A/C-T)	6 (2.9)
Pre-NAC clinical T stage	cT1	13 (6.2)
	cT2	104 (49.8)
	cT3	56 (26.8)
	cT4	36 (17.2)
Pre-NAC clinical N stage	cN0	12 (5.7)
	cN1	90 (43.1)
	cN2	76 (36.4)
	cN3	31 (14.8)
Pre-NAC tumor size, cm	Mean (±sd)	4.74 (±2.5)
Post-NAC tumor size, cm	Mean (±sd)	2.41 (±2.3)
Reduction rate after NAC, %	Mean (±sd)	48.2 (±37.8)
Response to NAC	Reduction rate ≥10%	184 (88.0)
	Reduction rate <10%	25 (12.0)
pCR	Yes	15 (7.2)
	No	194 (92.8)

sd, Standard deviation; IDC, invasive ductal carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; p-AKT, phosphorylated AKT; p-S6K1, phosphorylated S6K1; NAC, neoadjuvant chemotherapy; BCS, breast-conserving surgery; D, docetaxel; A, doxorubicin; E, epirubicin; C, cyclophosphamide; T, paclitaxel; pCR, pathologic complete response.

groups (p=0.003 and 0.042, respectively) compared to the p-AKT-negative and p-S6K1-negative groups. There were no significant differences in pre-NAC clinical T or N stages according to p-AKT or p-S6K1 expression status. However, there was a positive correlation between p-AKT and p-S6K1 expression status. Among p-AKT-positive tumors, 62.1% (41/66) were positive for p-S6K1, whereas 35.8% (48/134) of p-AKT-negative tumors were positive for p-S6K1 (p<0.001; data not shown). Similarly, 46.1% (41/89) of p-S6K1-positive tumors were positive for p-AKT, whereas 22.5% (25/111) of p-S6K1-negative tumors were positive for p-AKT (p<0.001; data not shown).

Correlation between clinicopathological variables and response to NAC. To identify the factors predicting for response to NAC, various clinicopathological parameters were analyzed (Table III). In this analysis, p-S6K1 expression status significantly predicted response to NAC (p=0.014). Out of NAC-resistant tumors (<10% reduction), 68.0% were p-S6K1-positive. In contrast, 41.8% of tumors that showed a minimum 10% reduction following NAC were p-S6K1-positive. Multivariate regression analysis was performed to identify the factors predicting for NAC resistance (Table IV). This showed that p-S6K1 expression status was the only significant predictive factor; p-S6K1 positivity was associated with an approximately four-fold higher likelihood of <10% tumor reduction after NAC (hazard ratio=3.81; 95% confidence interval=1.28-11.31; p=0.016).

Discussion

In this clinical report, we examined the relationship between the PI3K/AKT/mTOR pathway and response to NAC. We assessed 209 patients with LABC who received NAC and found that the expression status of p-S6K1, a key effector of the PI3K/AKT/mTOR pathway, is associated with chemoresponsiveness, that is, tumors expressing p-S6K1 were more resistant to NAC.

The correlation between the PI3K/AKT/mTOR pathway and resistance to endocrine therapy is well-known. This pathway modulates response to signals communicated through the ER and is important in clinical sensitivity to endocrine therapy (22-25). Recent clinical trials in the neoadjuvant (RAD2222) and metastatic setting (TAMRAD and BOLERO-2) have reported improved clinical outcomes in patients with ER-positive breast cancer when mTOR inhibitors are added to standard endocrine therapy (26-28). Previously, we reported that the expression of p-S6K1 could be a possible marker for resistance to endocrine therapy (21).

However, compared with endocrine therapy, limited evidence is available on the association of the PI3K/AKT/mTOR pathway with chemotherapy resistance. Several studies have reported that rapamycin and its analogs enhance the efficacy of cytotoxic agents in several cancer cell types, including breast cancer (29-33). In ovarian cancer, taxanes interacted with this signaling pathway to promote apoptosis, and AKT activation promoted taxane resistance (17, 18). In prostate cancer, high expression of p-AKT and p-S6K1 were associated with doxorubicin resistance, and

Table II. *Characteristics according to p-AKT and p-S6K1 expression.*

Variable		p-AKT, n (%)			p-S6K1, n (%)		
		Negative	Positive	*p*-Value	Negative	Positive	*p*-Value
Age, years	<50	64 (47.8)	30 (45.5)	0.759	49 (50.0)	49 (50.0)	0.170
	≥50	70 (52.2)	36 (54.5)		66 (59.5)	45 (40.5)	
Histopathological type	IDC	121 (90.3)	60 (90.9)	0.890	104 (90.4)	85 (90.4)	0.998
	Other	13 (9.7)	6 (9.1)		11 (9.6)	9 (9.6)	
Grade	1/2	51 (53.7)	28 (70.0)	0.079	53 (62.4)	28 (50.9)	0.180
	3	44 (46.3)	12 (30.0)		32 (37.6)	27 (49.1)	
ER	Negative	69 (51.5)	29 (43.9)	0.315	54 (47.0)	50 (53.2)	0.370
	Positive	65 (48.5)	37 (56.1)		61 (53.0)	44 (46.8)	
PR	Negative	70 (52.2)	20 (30.3)	0.003	59 (51.3)	35 (37.2)	0.042
	Positive	64 (47.8)	46 (69.7)		56 (48.7)	59 (62.8)	
HER2	Negative	81 (60.4)	38 (57.6)	0.697	75 (65.2)	51 (54.3)	0.107
	Positive	53 (39.6)	28 (42.4)		40 (34.8)	43 (45.7)	
Pre-NAC tumor size, mean (±sd), cm		4.64 (±2.65)	5.08 (±2.25)	0.214	4.58 (±2.40)	4.93 (±2.63)	0.299
Pre-NAC clinical T stage	1	9 (6.7)	4 (6.1)	0.609	11 (9.6)	2 (2.1)	0.075
	2	66 (49.3)	30 (45.5)		53 (46.1)	51 (54.3)	
	3	33 (24.6)	22 (33.3)		34 (29.6)	22 (23.4)	
	4	26 (19.4)	10 (15.2)		17 (14.8)	19 (20.2)	
Pre-NAC clinical N stage	0	6 (4.5)	5 (7.6)	0.097	7 (6.1)	5 (5.3)	0.412
	1	58 (43.3)	27 (40.9)		54 (47.0)	36 (38.3)	
	2	44 (32.8)	29 (43.9)		36 (31.3)	40 (42.6)	
	3	26 (19.4)	5 (7.6)		18 (15.7)	13 (13.8)	

p-AKT, Phosphorylated AKT; p-S6K1, phosphorylated S6K1; IDC, invasive ductal carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; sd, standard deviation; NAC, neoadjuvant chemotherapy.

mTOR inhibitors reversed doxorubicin resistance (19). Similarly, in head and neck cancer cells, addition of the mTOR inhibitor prevented S6K1 phosphorylation and restored sensitivity to doxorubicin (34). In breast cancer, AKT activation by introduction of a constitutionally activated *AKT1* gene resulted in resistance to chemotherapeutic drugs (35). Recently, Yi *et al*. reported the synergism of PI3K/AKT/mTOR pathway inhibition and chemotherapeutic drugs in breast cancer-1 gene (*BRCA1*)-defective breast cancer cells (36). Although pre-clinical data and our results suggest a correlation between the PI3K/AKT/mTOR pathway and chemoresistance, and also that mTOR inhibitors may reverse chemotherapy resistance, the mechanism of this correlation is not well-understood. The cellular targets of chemotherapeutic agents such as anthracyclines, taxanes and mTOR inhibitors are different; therefore, further studies are needed to elucidate the mechanism by which the PI3K/AKT/mTOR pathway influences chemotherapy resistance.

In breast cancer, NAC achieves a clinical response in 60-90% of patients (37). A pCR after NAC occurs in 3–16%, which is regarded as a predictor of survival (3-5, 37-40). Patients who achieve pCR are the most obvious beneficiaries of NAC, and thus, most studies have sought to determine clinical and molecular predictors of pCR. However, identifying factors predicting resistance to NAC may be just as important as identifying pCR predictors, and may play an even more important role in patient management. The classification of patients that have little or no response to NAC is important because these patients could be spared the toxicity of ineffective therapy and instead be guided towards alternative therapies. Furthermore, the identification of molecular markers predicting chemotherapy resistance could give an insight into mechanisms of resistance and may lead to the development of novel targeted agents that enhance sensitivity to chemotherapy in selected patients.

Caudle *et al*. assessed 1,928 patients and reported that African-American race, advanced stage, high grade, high Ki-67, and ER/PR negativity were factors predictive of disease progression during NAC (41). However, some of these factors, such as ER/PR negativity, high grade, and high Ki-67, have also been shown to be predictors of a greater response to chemotherapy (42, 43), which suggests that morphologically similar and aggressive tumors may represent two different sub-populations: one highly sensitive to chemotherapy and another highly resistant. Clinicopathological features alone cannot be used for differentiating between them. Thus, novel molecular markers

Table III. *Correlation of various parameters with response to neoadjuvant chemotherapy.*

Variable		No. of patients (%)		*p*-Value
		Responder (≥10% reduction)	Non-responder (<10% reduction)	
Age, years	<50	86 (46.7)	12 (48.0)	0.906
	≥50	98 (53.3)	13 (52.0)	
Histopathological type	IDC	169 (91.8)	20 (80.0)	0.059
	Other	15 (8.2)	5 (20.0)	
Grade	1/2	71 (58.7)	10 (52.6)	0.620
	3	50 (41.3)	9 (47.4)	
ER	Negative	91 (49.5)	13 (52.0)	0.811
	Positive	93 (50.5)	12 (48.0)	
PR	Negative	85 (46.2)	9 (36.0)	0.336
	Positive	99 (53.8)	16 (64.0)	
HER2	Negative	111 (60.3)	15 (60.0)	0.975
	Positive	73 (39.7)	10 (40.0)	
p-AKT	Negative	121 (69.1)	13 (52.0)	0.088
	Positive	54 (30.9)	12 (48.0)	
p-S6K1	Negative	107 (58.2)	8 (32.0)	0.014
	Positive	77 (41.8)	17 (68.0)	

NAC, Neoadjuvant chemotherapy; IDC, invasive ductal carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; p-AKT, phosphorylated AKT; p-S6K1, phosphorylated S6K1.

for predicting chemotherapy response need to be developed.

In the current study, PR expression was positively correlated with p-AKT and p-S6K1 expression status. The link between PR and the PI3K/AKT/mTOR pathway is not well-understood. Several studies have reported that PR expression is inhibited *via* PI3K/AKT/mTOR pathway activation (44, 45). mRNA expression analysis also showed enrichment of up-regulated genes in the PI3K/AKT/mTOR pathway in both ER-positive/PR-negative and ER-negative/PR-negative tumors compared to ER-positive/PR-positive tumors (46). In contrast, other studies have shown a positive correlation between PI3K/AKT/mTOR pathway activation and PR expression (47, 48).

Our study is limited by its retrospective nature. The study population did not receive homogeneous treatment. While most patients received docetaxel plus anthracycline for their NAC regimen, a few patients received sequential regimens such as doxorubicin and cyclophosphamide followed by taxane. In addition, the number of NAC cycles was not homogeneous. In this study, only 31 patients (14.8%) received more than four NAC cycles, and the remaining 178 patients (85.2%) received three NAC cycles (data not shown). The small number of cases with extended NAC cycles may have resulted in the paucity of pCR cases (7.2%) in the present study. More importantly, the modalities assessing tumor size before and after NAC were different: the pre-NAC tumor size was measured by imaging, whereas the post-NAC tumor size was evaluated by pathological examination.

Table IV. *Results of multivariate analysis of factors predicting resistance to neoadjuvant chemotherapy.*

Variable	Hazard ratio	95% confidence interval		*p*-Value
		Lower	Upper	
Age (<50 years *vs*. ≥50 years)	1.20	0.42	3.42	0.737
Histopathology (IDC *vs*. other)	0.20	0.04	1.11	0.066
Grade (3 *vs*. 1/2)	1.56	0.47	5.18	0.470
ER (positive *vs*. negative)	1.02	0.29	3.64	0.972
PR (positive *vs*. negative)	1.27	0.38	4.21	0.694
HER2 (positive *vs*. negative)	0.71	0.23	2.12	0.535
p-AKT (positive *vs*. negative)	1.80	0.60	5.42	0.297
p-S6K1 (positive *vs*. negative)	3.81	1.28	11.31	0.016

IDC, Invasive ductal carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; p-AKT, phosphorylated AKT; p-S6K1, phosphorylated S6K1.

Chemotherapy resistance is a major clinical problem in the treatment of breast cancer. Two major challenges for successful chemotherapy may be the development of more specific markers to predict response and the development of novel targeted agents that would enhance sensitivity to chemotherapy in selected patients. Our results suggest that the PI3K/AKT/mTOR pathway may be exploited, allowing S6K1 to be utilized as a marker predictive of resistance and as a

therapeutic target for enhancing sensitivity to chemotherapy in selected patients. Determination of the mechanism by which the PI3K/AKT/mTOR pathway influences chemoresponsiveness will aid in the identification of patients who will benefit most from this therapeutic approach.

Acknowledgements

This research has been supported by a grant from the Radiological Translational Research Program (50451-2013).

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Received June 30, 2013
Revised July 16, 2013
Accepted July 17, 2013