Relationship between Ribosomal Protein S6-pS240 Expression and other Prognostic Factors in Non-Special Type Invasive Breast Cancer

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Keywords

Ribosomal protein S6-pS240 · Breast cancer ·
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Summary

Background: The aim of this study was to investigate the immunohistochemical expression of ribosomal protein (RP) S6-pS240 in non-special type invasive breast cancer in relation to other prognostic markers and gain new insights to facilitate more individualized treatment. **Methods:** The following clinical and histopathological parameters of 120 patients were determined: S6-pS240 expression, age, menopausal status, tumor size and grade, TNM stage, Nottingham Prognostic Index (NPI), lymph node stage, estrogen and progesterone receptor (ER/PR) expression, HER2/neu amplification, lymphovascular invasion, and proliferative index as measured by Ki-67. Treatment protocol and disease-free survival were evaluated accordingly. **Results:** Significant positive correlations were seen between S6-pS240 expression and Ki-67 values ($\rho = 0.530$, $p < 0.001$), and NPI ($\rho = 0.370$, $p < 0.001$) and HER2/neu amplification ($\rho = 0.368$, $p < 0.001$). A negative correlation was found between S6-pS240 and ER/PR expression ($\rho = 0.362$, $p < 0.001$). Patients with negative RP S6-pS240 expression had significantly longer disease-free survival (log-rank test, $p = 0.005$). **Conclusion:** Immunohistochemical analysis of RP S6-pS240 is a valuable additional prognostic marker in patients with invasive breast cancer. Routine use of S6-pS240 immunohistochemistry is recommended.

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Introduction

Breast cancer of no special type (NST) is one of the most common malignancies worldwide. Personal history, environmental factors, as well as hormonal and reproductive status are thought to be related to it [1]. Breast cancer has a mortality incidence ratio of 0.4, ranking 5th as a cause of cancer-related death. A relatively favorable prognosis and breast screening programs carried out in most developed countries contribute to these statistics [2].

There has been an important change in the surgical procedures used for breast cancer treatment, from radical mastectomy to breast-conserving surgery [3, 4]. Local control is the major objective of surgical treatment and part of systemic therapy, and breast-conserving surgery is the mainstay of treatment. About one-third of women with breast cancer still undergo mastectomy, depending on the size or the site of the lesion and the presence of an extensive intraductal component [5].

Numerous established prognostic markers of breast cancer (NST) exist, such as grade of differentiation, tumor size, regional lymph node status, histological type, proliferation index, and HER2/neu amplification or expression of hormone receptors [6–9]. Multiple unfavorable genetic events and subsequent molecular alterations may trigger tumor progression. The human ribosomal protein S6 (RPS6) gene is located at chromosome 9p21. It consists of 6 different exons and is 3,979 base pairs long [10]. It encodes a cytoplasmic ribosomal protein (RP) that is a component of the ribosomal 40S subunit. Multiple pseudogenes of RPS6 are dispersed throughout the genome. The protein, product of RPS6 gene transcription, belongs to the S6E family of RPs. Lack of RPS6 is related to absence of nascent ribosomal units, as recent experiments have shown. Loss of RPS6, on the other hand, may lead to an abundance of other RPs and activation of extra-ribosomal functions of these proteins [11].

Table 1. Differences in clinical variables regarding ribosomal protein (RP) S6-pS240 expression groups: chi-square and Mann-Whitney U test

	RP S6-pS240		p
	no expression (0) (n = 66)	expression (1,2,3) (n = 54)	
T status, n (%)			
T1	28 (42.4)	13 (24.1)	0.103
T2	33 (50.0)	34 (63.0)	
T3	4 (6.1)	7 (13.0)	
T4	1 (1.5)	0 (0.0)	
N status, n (%)			
N0	41 (62.1)	18 (33.3)	0.006
N1	12 (18.2)	13 (24.1)	
N2	7 (10.6)	7 (13.0)	
N3	6 (9.1)	16 (29.6)	
Positive metastasis, n (%)	2 (3.0)	12 (22.2)	0.001
Death outcome, n (%)	3 (4.5)	3 (5.6)	0.801
Surgical treatment, n (%)			
Simple mastectomy	4 (6.1)	3 (5.6)	0.352
Radical mastectomy	55 (83.3)	49 (90.7)	
Lumpectomy + axillary dissection	7 (10.6)	2 (3.7)	
Other treatment, n (%)			
Chemotherapy	64 (97.0)	54 (100.0)	0.197
Hormone therapy	59 (89.4)	35 (64.8)	0.001
Herceptin therapy	5 (7.6)	19 (35.2)	0.020
Radiotherapy	44 (66.7)	46 (85.2)	0.020
Bisphosphonate therapy	6 (9.1)	13 (24.1)	0.025
Triple negative, n (%)	4 (6.1)	5 (9.3)	0.508
Age, median (IQR), years ^a	57.5 (45.8–67.3)	49.0 (43.0–65.0)	0.053
Nottingham Prognostic Index, median (IQR) ^a	3.4 (2.5–4.6)	5.1 (4.4–6.4)	<0.001
Survival time, median (IQR), months ^a	33.1 (22.4–50.6)	32.4 (22.7–43.3)	0.746
Estrogen receptor, median (IQR) ^a	12.0 (6.0–12.0)	6.0 (0.0–12.0)	0.001
Progesterone receptor, median (IQR) ^a	12.0 (6.0–12.0)	1.5 (0.0–12.0)	<0.001
HER2/neu, median (IQR) ^a	0.0 (0.0–1.0)	1.0 (0.0–3.0)	<0.001
Ki-67, median (IQR) ^a	0.05 (0.03–0.07)	0.30 (0.06–0.60)	<0.001

^aMann-Whitney U test; IQR = interquartile range.

There is a lack of understanding of the immunohistochemical detection of phosphorylated RP S6-pS240 in breast cancer tissue. In breast cancer, RPS6 hyperphosphorylation and increased activity have been reported [12–14]. The investigation of the immunohistochemical expression of RP S6-pS240 in correlation with other prognostic markers can potentially contribute valuable prognostic and predictive insight and may provide an additional prognostic marker in patients with breast cancer.

Materials and Methods

Study Groups

Paraffin blocks from 120 female patients diagnosed with breast cancer (NST) were retrieved from our archive. Patients had been treated with modified radical mastectomy at the Clinic of Thoracic Surgery, University Clinical Center of Kosovo during the period of 2006–2012. Tumor-node-metastasis (TNM) staging had been employed accordingly. The following parameters were determined for each patient: age, menopausal status, tumor size, histological grade, regional lymph node stage, TNM, Nottingham Prognostic Index (NPI), hormone receptor status, HER2/neu amplification, proliferative index as measured by Ki-67, and expression of RP S6-pS240. Treatment protocol and disease-free survival were evaluated accordingly. Histological grade was determined using the Nottingham grading system [6, 15]. Cases subjected to neoadjuvant

therapy and with a multifocal and/or multicentric component were excluded from the study. Follow-up was carried out in accordance with the guidelines of the American Society of Clinical Oncology (ASCO) on breast cancer [16]. These were: physical examination every 3 months for 3 years, mammography every 6 months, and first post-treatment mammogram 1 year after the initial mammogram and no earlier than 6 months after definitive radiation therapy.

Immunohistochemistry

Biopsy samples were fixed in 10% neutral buffered formalin and sectioned in 3–4 micron thick sections. All biopsy samples had been previously evaluated by 2 independent pathologists. Immunohistochemical analysis was carried out manually. Antibody clone, origin, supplier, pretreatment, and dilution are presented in table 1. Antigen retrieval was carried out by placing the slides in target retrieval solution for 45 min at 95–98 °C. The slides were incubated with the primary antibody for 30 min. Visualization was carried out with dextran polymer conjugated with peroxidase and secondary antibody (EnVision+, DAKO, Glostrup, Denmark, K534011) for 30 min. Estrogen receptor (ER), progesterone receptor (PR), and HER2/neu immunohistochemistry were carried out following the ASCO guidelines [17–19].

A positive result for ER and PR was any nuclear stain observed in more than 1% of the tumor cells (fig. 1). Non-neoplastic epithelium of the normal terminal duct-lobular unit was used as an internal control. The staining pattern for HER2/neu was scored on a scale of 0–3+. Negative for HER2/neu overexpression were scores of 0 and 1+. Positive results were membranous staining with a score of 3+. Cases with ‘equivocal’ results (2+) were excluded to restrict immunohistochemical methodology.

Fig. 1. **A, B** Mild, **C, D** moderate, and **E, F** strong immunoreactivity of tumor cells for S6-pS240 (immunoperoxidase stain, $\times 10$, $\times 20$, and $\times 40$ magnification).

Interpretation of the proliferative index as measured by Ki-67 was carried out by estimating the percentage of cells with nuclear stain in the most mitotically active areas. The cytoplasmic staining pattern for S6-pS240 was scored on a scale of 0–3+. The cut-off value of RP S6-pS240 expression was set according to ROC curve analysis in relation to disease-free outcomes during the monitored time (values > 0 : area under the curve (AUC) 0.75; $p = 0.001$, sensitivity 85.7% (95% confidence interval (CI) 57.2–98.2%), and specificity 60.4% (95% CI 50.4–69.7%) with a positive predictive value (PV) of 22.2 (95% CI 17.2–28.2) and negative PV of 97.0 (95% CI 89.8–99.1). Results showed satisfactory transferability as well as intra- and inter-laboratory reproducibility.

Statistical Methods

Normality of data distribution was assessed with the Smirnov-Kolmogorov test, and according to results appropriate non-parametric tests were used in analysis. Differences between RP S6-pS240 expression groups were analyzed with the χ^2 test (categorical values) and Mann-Whitney U test (quantitative values). Spearman's correlation coefficients were used to analyze associations between RP S6-pS240 expression and other clinical variables (table 2). A Kaplan-Meier survival curve with corresponding log-rank test was made to analyze RP S6-pS240 expression in relation to disease recurrence outcome. Binary logistic regression was performed to assess the multivariate impact of factors on the likelihood of patients having positive RP S6-pS240 expression. All p values below 0.05 were considered significant. The data analysis software system IBM SPSS Statistics, version 25.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis and graphic images.

Results

Positive statistical correlation was demonstrated between expression of RP S6-pS240 and higher values of N and M stage and NPI value, especially in groups with NPI values over 5.40 and HER2/neu amplification. Patients with positive expression of RP S6-pS240

Table 2. Spearman's correlation coefficients between clinical variables and ribosomal protein (RP) S6-pS240 expression

	RP S6-pS240
T	
Rho	0.144
P	0.116
N	120
N	
Rho	0.277
P	0.002
N	120
M	
Rho	0.248
P	0.006
N	120
Nottingham Prognostic Index	
Rho	0.370
P	< 0.001
N	120
Estrogen receptor	
Rho	–0.362
P	< 0.001
N	120
Progesterone receptor	
Rho	–0.363
P	< 0.001
N	120
HER2/neu	
Rho	0.368
P	< 0.001
N	120
Ki-67	
Rho	0.530
P	< 0.001
N	120
Survival time, months	
Rho	–0.014
P	0.882
N	120
Age, years	
Rho	–0.160
P	0.082
N	120

were more likely to be subjected to radio- and bisphosphonate therapy. In contrast, lack of expression of RP S6-pS240 coincided with administration of hormonal therapy. Cases with positive RP S6-pS240 expression had significantly higher median values of NPI and Ki-67 and were more likely to be HER2/neu amplified. In contrast, cases without RP S6-pS240 had higher median values of ER and PR, indicating better outcome. Significant positive correlations were observed between RP S6-pS240 expression and higher N and M stage, NPI value, HER2/neu amplification, and Ki-67 index. A negative correlation was observed with ER/PR expression. The highest positive correlation coefficient ($\rho = 0.530$, $p < 0.001$) was observed with Ki-67. Figure 2 shows the Kaplan-Meier survival curve regarding RP S6-pS240 expression in relation to disease recurrence outcome. The log-rank test was significant ($p = 0.005$), indicating that patients with negative RP S6-pS240 expression had significantly longer disease-free survival. The binary regression model contained 9 independent variables (ER, PR, HER2/neu, Ki-67, NPI value, age, and TNM stage). The full model containing all predictors was statis-

Fig. 2. Kaplan-Meier survival curve regarding ribosomal protein S6-pS240 expression (log-rank test: $p = 0.005$).

Table 3. Prediction of positive ribosome protein S6-pS240 expression: binary logistic regression

	B	Standard error	Odds ratio	95% confidence interval		p
				lower	upper	
ER	0.00	0.06	1.00	0.88	1.14	0.982
PR	-0.04	0.06	0.96	0.85	1.08	0.487
HER2/neu	0.34	0.24	1.41	0.88	2.24	0.150
Ki-67	3.33	1.14	27.95	2.98	261.97	0.004
NPI group	-0.06	0.53	0.94	0.33	2.65	0.905
Age, years	-0.01	0.02	0.99	0.95	1.03	0.540
T	-0.63	0.55	0.53	0.18	1.55	0.248
N	0.35	0.32	1.42	0.75	2.68	0.275
M	1.35	0.91	3.85	0.64	22.97	0.140

ER = Estrogen receptor; PR = progesterone receptor; NPI = Nottingham Prognostic Index.

tically significant ($p < 0.001$), indicating that the model was able to distinguish between patients who have positive and negative RP S6-pS240 expression. The model explained 40.1% of the variance in RP S6-pS240 expression status and correctly classified 77% of cases. As shown in table 3, only Ki-67 as an independent variable made a unique statistical contribution to the model. This indicates that patients with higher Ki-67 values were nearly 28 times more likely to have positive RP S6-pS240 expression.

Discussion

In this study, we observed significant positive correlations between RP S6-pS240 expression and higher N and M stage, NPI value, HER2/neu amplification, and Ki-67 index. A significant negative correlation was observed with ER/PR expression. Numerous

unfavorable genetic events and subsequent molecular cell changes may trigger tumor progression. The human *RPS6* gene encodes a cytoplasmic RP that is a component of the ribosomal 40S subunit. Loss of RPS6 expression can lead to an abundance of other RPs and activation of extra-ribosomal functions of these proteins, as recent studies have shown [11, 20, 21].

RPS6 is the major substrate of protein kinases in the ribosome, with subsets of 5 C-terminal serine residues (pS235, pS236, pS240, pS244, and pS247), phosphorylated by many different protein kinases. The focus of research are S6 kinases 1 and 2 (S6K1, S6K2). A wide range of stimuli, such as growth factors, tumor-promoting agents, and mitogens, induce phosphorylation [20–22]. Dephosphorylation occurs in growth arrest. Through selective translation of classes of mRNA, TOP (terminal oligopyrimidine tract in the 5'UTR-untranslated region) mRNA-encoded RPs and elongation factors (EF1 and EF2) may contribute to the control of cell growth and proliferation [23, 24]. Some proteins encoded by TOP genes can act as proto-oncogenes [25]. Conditional deletion of *RPS6* results in ribosome biogenesis interruption and progression of cell cycle, as experiments using mice have shown [26].

It has been observed that soon after cloning of these enzymes, their activity is highly sensitive to the antifungal agent rapamycin. Inhibition of mTOR (mammalian target of rapamycin) with rapamycin causes a drastic reduction in RPS6 phosphorylation in mammalian cells. The impact of the mTOR/S6K signaling cascade is important, considering that mTOR and/or S6K phosphorylate downstream targets (RPS6 and many other proteins as well), which in turn leads to the modulation of different cellular functions: cell migration, survival, growth, and proliferation [20–22, 25, 26]. Increased activity of mTOR, S6K, and RPS6 as well as hyperphosphorylation have been found in different cancer types (neuroblastoma, esophageal, cervical, hematologic, hepatocellular, and breast malignancies, many types of sarcoma, etc.) [12, 13, 27].

The value of the immunohistochemical detection of phosphorylated RP S6-pS240 in breast cancer has not been thoroughly studied. Since most reports focus on RPS6 hyperphosphorylation and increased activity in breast cancer [12, 14, 28], the investigation of RP S6-pS240 in breast cancer may potentially have valuable prognostic and predictive implications. Hence, the immunohistochemical analysis of RP S6-pS240 may provide an additional prognostic marker in patients with breast cancer.

In the study by Yanai et al. [29] it was observed that pS6 expression level is associated with characteristics of breast cancer with high Ki-67 expression. Given these associations, irrespective of menopausal status, the biological difference seems to be less affected by estrogen signaling than by S6 protein activation, especially in terms of proliferation. The authors indicated that targeting the mTOR/S6K pathway might be a useful strategy for the treatment of ER+/HER2/neu– breast cancer with high Ki-67 expression. Potemski et al. [30] demonstrated that Ki-67 expression levels measured by immunostaining and real-time reverse transcription polymerase chain reaction were weakly concordant, and both were, while not influencing survival, related to higher tumor grade. Our study results support these findings.

Limitations of this Study

Although this study met its targets, there are some inevitable limitations. Due to financial and ethical reasons, we did not include a healthy control group to explore RP S6-pS240 expression and compare it with the breast cancer (NST) group. Future studies with longer monitoring time, other types of breast cancer, and more participants are recommended.

Conclusion

Immunohistochemical analysis of RP S6-pS240 is a valuable additional prognostic marker in patients with invasive breast cancer (NST). RP S6-pS240 significantly correlates with other established prognostic parameters, especially Ki-67. Ki-67 as an independent variable made a unique statistical contribution to the binary regression model of disease recurrence: Patients who had higher Ki-67 values were nearly 28 times more likely to have positive RP S6-

pS240 expression. In terms of therapy, study subjects with positive expression of RP S6-pS240 were more likely to have received radiotherapy and bisphosphonates.

Patients with negative RP S6-pS240 expression had significantly longer disease-free survival. These findings offer new perspectives for better individual treatment.

Approval by the Research Ethics Committee

This study is conducted in compliance with the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989, and 1996 and approved by the Ethics and Professionalism Committee.

Disclosure Statement

None of the authors have any conflicts of interest in relation to this submission.

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