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The mTOR Signaling Pathway Is Associated With the Prognosis of Malignant Pleural Mesothelioma After Multimodality Therapy

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Abstract. *Background/Aim: We performed multimodality therapy comprising preoperative chemotherapy, extrapleural pneumonectomy (EPP), and radiation therapy for patients with malignant pleural mesothelioma (MPM). Although multimodality therapy resulted in good prognosis, further improvement is required. Therefore, herein, we analysed the prognostic factors using surgical specimens and searched for suitable molecular targets to improve the prognosis after multidisciplinary treatment. Patients and Methods: Forty-six patients with MPM underwent multimodality therapy. Paraffin-embedded surgical samples were used for immunohistochemistry to evaluate the expression of phosphorylated (p-) AKT, extracellular signal-regulated kinase (ERK), mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), and S6 ribosomal protein (S6RP). Results: On univariate and multivariate analyses, significant differences were observed according to the histological type, pathological stage, and p-mTOR expression rate. Conclusion: The prognosis of MPM is affected by p-mTOR expression, suggesting that molecular-targeted treatment might be used during multimodal therapy for MPM.*

Malignant pleural mesothelioma (MPM) is a rare disease (1-3), and the median survival of untreated patients is 4-12 months (4-6). The occurrence of MPM is strongly associated with asbestos exposure, and MPM develops after a latent period of 20-40 years (2-7). First-line therapy consists of combination therapy using cisplatin and pemetrexed, which results in better outcomes compared to the outcomes after cisplatin monotherapy (8, 9). However, cisplatin and pemetrexed combination therapy only extends the median survival by 9-12 months (8). Therefore, multimodal therapy comprising preoperative chemotherapy, extrapleural pneumonectomy (EPP), and radiation therapy is currently performed (10, 11). Although multimodal therapy results in good results, with an overall median survival of 19.9 months and a 2-year survival rate of 42.9%, EPP leaves minute tumors on the surface of the resected stump due to the irregular nature of chest wall detachment (12). Therefore, postoperative chemotherapy using cisplatin and pemetrexed or radiotherapy is required. In addition, as there are only a few effective anticancer drugs for MPM, new drugs are needed to improve the prognosis of patients with MPM.

The mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways play important roles in responding to extracellular signals from cytokines and growth factors, as well as regulating cell function (13, 14); these pathways are also known to be overactivated in some sarcomas (15). While BRAF inhibitors and mitogen-activated protein kinase kinase (MEK) inhibitors targeting the MAPK pathway, and mTOR inhibitors targeting the phosphatidylinositol-4,5-*bis*phosphate 3-kinase (PI3K)/AKT serine/threonine kinase (AKT)/mTOR pathway are currently in use, only a few studies have evaluated the extent of overactivation of these pathways in MPM cases.

Therefore, the current study investigated the possibility of using molecular-targeted drugs as multimodal therapy for MPM by analyzing the correlation between AKT/mTOR and

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MAPK pathways and prognosis of MPM cases treated with multimodal therapy including EPP.

Patients and Methods

Patients and treatment. Multimodal therapy including EPP was administered to 46 patients with MPM at our Department between April 2004 and October 2012. After the diagnosis of MPM on pleural biopsy, three courses of chemotherapy (75 mg/m^2 cisplatin and 500 mg/m^2 pemetrexed every 21 days) were administered and EPP was performed for patients who did not show any disease progression. The same chemotherapy was continued after surgery. The tumors were staged using the International Mesothelioma Interest Group (IMIG) staging system (16). This study was approved by The Ethics Review Board for Human Genome/Gene Analysis Research, Hyogo College of Medicine (no. 0044), and informed consent was obtained from each patient prior to evaluating the specimens.

Immunohistochemistry. Immunohistochemistry was performed using intra-operatively obtained paraffin-embedded tissue samples. Formalin-fixed specimens were embedded in paraffin and cut into 3-μm slices. They were then immersed in a buffer solution of pH 9 (DAKO, Carpinteria, CA, USA) and kept at 98˚C for 20 minutes before being cooled for 20 minutes to stimulate the antigens. The following rabbit monoclonal antibodies were used as primary antibodies: phosphorylated (*p*-) AKT (Ser473; 1:50), p-mammalian target of rapamycin (mTOR) (Ser2448; 1:100), p-S6 ribosomal protein (S6RP) (Ser240/244; 1:2000, 1:1600), p-eukaryotic translation initiation factor 4E-binding protein 1 (4E–BP1) (Thr37/46; 1:400), p-MEK (Ser221; 1:50), and p-ERK (Thr202/Tyr204; 1:400) (Cell Signaling Technology, Danvers, MA, USA). A DAKO Autostainer (Ft. Collins, CO, USA) was used for staining. The expression of p-AKT, p-mTOR, p-S6RP, p-4E-BP1, p-MEK and p-ERK were analyzed by the intensity of staining by two researchers who were blinded to the patients' baseline characteristics.

Statistical analysis. Continuous and categorical variables are presented as the median values with ranges and as frequencies with percentages, respectively. The curves for overall survival from the time of surgery were estimated using the Kaplan–Meier product-limit method, and were compared using the log-rank test for univariate analysis. Factors that were found to have values of $p<0.1$ on univariate analysis were included in the Cox proportional-hazards regression model for multivariate analysis. The results are presented as the hazards ratios (HRs), 95% confidence intervals (CI), and *p*-values. All *p*-values were two-sided, and values of $p<0.05$ were considered to indicate statistical significance. Statistical analyses were performed using SPSS Statistics software (version 15.0; SPSS Inc., Chicago, IL, USA).

Results

Multimodal therapy including EPP was administered to 46 patients with MPM: 35 men (76.1%) and 11 women (23.9%), with a mean age of 59.8 years (range=37-71 years). Histological type was epithelial in the majority of cases (93.5%). The disease stage was stage III or IV in most cases (71.7%) (Table I).

Table I. *Clinical characteristics of patients with malignant pleural mesothelioma (N=46).*

Characteristic	Value
Gender, n (%)	
Male	35 (76.1%)
Female	11 (23.9%)
Age, years	
Median	59.8 (37-71)
Histology, n (%)	
Epithelial type	43 (93.5%)
Biphasic type	2 (4.3%)
Sarcomatoid type	1 (2.2%)
Side, n (%)	
Left	27 (58.7%)
Right	19 (41.3%)
IMIG pStage, n (%)	
1	2 (4.3%)
2	11 (23.9%)
3	28 (60.9%)
4	5 (10.9%)

IMIG: International Mesothelioma Interest Group; p-Stage: pathological stage.

The results of immunohistochemical staining are shown in Table II. While the rate of positive expression for p-AKT, p-mTOR, and p-ERK was approximately 40%, the rates for p-S6RP and p-MEK were relatively high, at 69.6% and 87.0%, respectively (Table II).

No correlation was observed between the prognosis of patients and the positivity for p-AKT, p-4EBP1, and p-ERK ($p=0.925$, $p=0.650$, and $p=0.647$, respectively). Although of borderline significance, the prognosis of the p-mTOR-positive group was better than that of the p-mTOR-negative group (median 37.1 *vs*. 14.4 months, $p=0.085$). In contrast, the prognosis of the p-MEK-negative group was better than that of the p-MEK-positive group (not reached *vs*. 17.4 months, $p=0.084$). Moreover, the prognosis of the p-S6RP-positive group was significantly better than that of the p-S6RP-negative group (43.6 *vs*. 14.4 months, $p=0.031$; Figure 1).

On univariate analysis, epithelioid histological type (vs. non-epithelioid) led to significantly poorer prognosis (HR=4.328, 95% CI=1.366-13.715; $p=0.013$). The prognosis was also significantly worse for those with p-stage IV (HR=3.698, 95% CI=1.231-11.115; $p=0.02$) and but better for those with positive p-S6RP expression (HR=0.435, 95% CI=0.2-0.946; $p=0.036$).

On multivariate analysis, only two out of the three above-mentioned factors remained significant: epithelioid histological type (HR=3.617, 95% CI=1.039-12.592; $p=0.043$), and p-Stage IV (HR=5.782, 95% CI=1.57-21.299; $p=0.008$) (Table III).

Table II. *Results of immunohistochemical staining (N = 46).*

Antigen	Protein name	Positive cases, n (%)	Negative cases, n (%)
AKT	Protein kinase B	18 (39.1%)	28 (60.9%)
mTOR	Mammalian target of rapamycin	18 (39.1%)	28 (60.9%)
S6RP	S6 ribosomal protein	32 (69.6%)	14 (30.4%)
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1	27 (58.7%)	19 (41.3%)
MEK	Mitogen-activated protein kinase kinase	40 (87.0%)	6 (13.0%)
ERK	Extracellular signal-regulated kinase	19 (41.3%)	27 (58.7%)

Discussion

The results of the current study showed that the prognosis of patients with MPM was influenced by p-mTOR expression, suggesting that molecular-targeted therapy has the potential to be used as part of multimodal therapy for MPM.

MAPK signaling cascades are linked to RAS, RAF, MEK, and ERK and play an important role in the proliferation of cells and resistance to anticancer drugs (17). Therefore, methods that block the MAPK pathway are being evaluated as a possible method for cancer treatment. The U.S. Food and Drug Administration has approved combination treatment with BRAF and MEK inhibitors such as dabrafenib and trametinib, vemurafenib and cobimetinib, and encorafenib and binimetinib for $BRAF^{V600}$-mutated advanced melanoma. The combination of dabrafenib and trametinib can be used after surgery for stage III melanoma. Vemurafenib was the first molecular-targeted drug to show a survival benefit for $BRAF^{V600E}$ metastatic melanoma. Vemurafenib was superior to dacarbazine when used as first-line treatment for $BRAF^{V600E}$-mutant metastatic melanoma. The objective response rate (ORR) after vemurafenib treatment was 48% *versus* 5% after dacarbazine treatment, while the median progression-free survival (PFS) rate was 5.3 *versus* 1.6 months, respectively (18).

Selective MEK inhibitors can inhibit the growth of *BRAF*- and *NRAS*-mutant melanoma. Trametinib was the first MEK inhibitor approved for the treatment of *BRAF*-mutated metastatic melanoma not previously treated with BRAF inhibitors. The ORR after trametinib treatment was 22% *versus* 8% after dacarbazine treatment, and the median PFS was 4.8 months *versus* 1.5 months for *BRAF*-mutant metastatic melanoma, respectively (19). Thus, MEK inhibitor therapy in combination with a BRAF inhibitor is more effective and less toxic than treatment with a BRAF inhibitor alone. In addition, when BRAF inhibitor or MEK inhibitor monotherapy is used, negative feedback through ERK1 and ERK2 can be an issue (20).

Approximately 50% of melanomas have *BRAF* mutations, with the V600E missense mutation accounting for approximately 80-90%. In 1,046 samples from patients who underwent radical surgery for primary non-small cell lung carcinoma, *BRAF* mutations were detected in 37 tumors (3.5%) and the *BRAF* mutation of V600E was observed in 56.7% of cases (21). Approximately 4% of MPM cases have *BRAF* mutations, and most of these mutations result in the substitution of *BRAF* V600E (22).

In the current study, the rate of positive expression of p-MEK was relatively high (87.0%), and the prognosis of the p-MEK-negative group was better than that of the p-MEK-positive group (not reached *versus* 17.4 months, $p=0.084$). In fact, for MPM, when the frequency of the *BRAF* V600E gene mutation is low (22), we expect MEK inhibitors to be effective because there are many p-MEK-positive cases.

In patients with mesothelioma, the level of hyaluronic acid (HA) in serum and pleural fluid is high, probably because HA is produced by both mesothelioma cells and normal mesothelial cells. HA, *via* receptors, modulates the intracellular signaling pathways, thereby resulting in cell proliferation, increased motility, and higher invasive properties of malignant cells (23). The inhibitor of HA synthesis 4-methylumbelliferone suppresses the growth of mesothelioma cell lines, and is effective for MPM treatment when combined with trametinib (24).

In the current study, the histological type, and p-Stage were prognostic factors after multidisciplinary treatment for MPM. In addition, p-mTOR expression was a prognostic factor on univariate analysis, and we propose a method that uses an mTOR inhibitor in combination with cisplatin/pemetrexed as a possible improvement. The first mTOR inhibitor to be discovered was rapamycin. At the time of discovery, rapamycin also showed immunosuppressive activity, and was therefore used as an immunosuppressant during organ transplantation. In addition, rapalogs including temsirolimus, everolimus, and ridaforolimus are currently used as anticancer agents, owing to the cytostatic effect of rapamycin on mTORC1 inhibition (25).

In a previous study, the p-mTOR positive rate was high in early-stage epithelial type mesothelioma and in the biphasic, sarcomatoid type, while the p-mTOR positive rate decreased in late stage MPM; in addition, overall survival tended to be

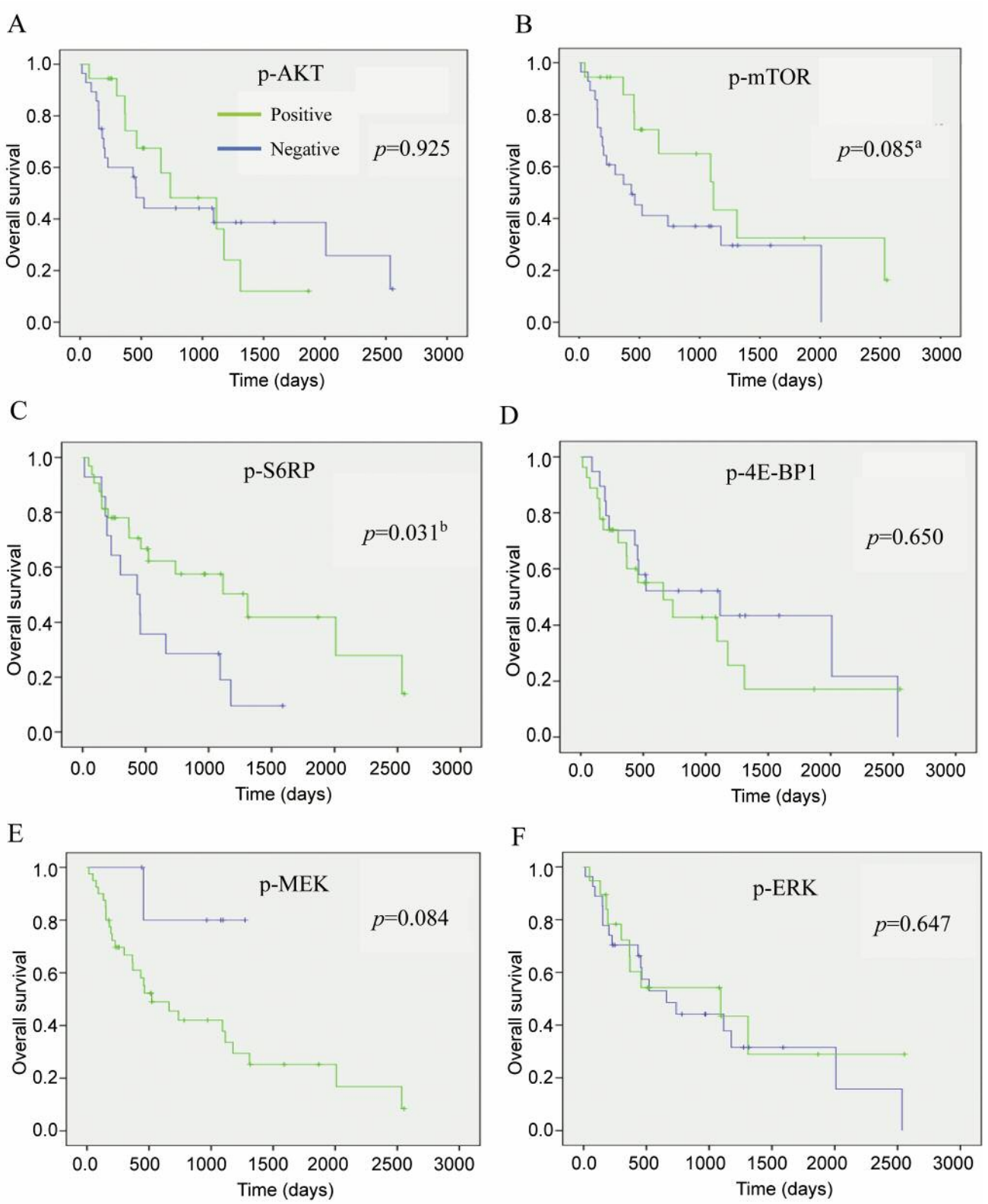

Figure 1. *Kaplan–Meier analysis of overall survival according to positivity for phosphorylated (p-) forms of protein kinase (p-AKT) (A), mammalian target of rapamycin (p-mTOR) (B), S6 ribosomal protein (p-S6RP) (C), eukaryotic translation initiation factor 4E-binding protein 1 (p-4E-BP1) (D), mitogen-activated protein kinase kinase (p-MEK) (E), and extracellular signal-regulated kinase (p-ERK) (F). p-Values when adjusted for multivariable factors: [a]p=0.107 and [b]p=0.314.*

Table III. *Univariate and multivariate analyses for preoperative factors in patients with malignant pleural mesothelioma (N=46).*

	Univariate analysis		Multivariate analysis	
Factor	HR 95% CI	*p*-Value	HR 95% CI	*p*-Value
Age				
>60 Years	1			
≤60 Years	0.416 (0.182-0.949)	0.037	0.716 (0.313-1.637)	0.428
Gender				
Male	1			
Female	0.453 (0.155-1.322)	0.147		
Side				
Left	1			
Right	0.847 (0.387-1.852)	0.677		
Histology				
Epithelioid	1		1	
Non-epithelioid	4.328 (1.366-13.715)	0.013	3.617 (1.039-12.592)	0.043
IMIG p-Stage				
I, II, III	1		1	
IV	3.698 (1.231-11.115)	0.02	5.782 (1.57-21.299)	0.008
p-S6RP				
Negative	1		1	
Positive	0.435 (0.200-0.946)	0.036	0.640 (0.268-1.525)	0.314
p-mTOR				
Negative	1		1	
Positive	0.489 (0.213-1.123)	0.091	0.471 (0.189-1.175)	0.107
p-MEK				
Negative	1			
Positive	4.921 (0.664-36.464)	0.119		
p-4EBP1				
Negative	1			
Positive	0.832 (0.375-1.845)	0.650		
p-AKT				
Negative	1			
Positive	0.954 (0.355-2.564)	0.925		
p-ERK				
Negative	1			
Positive	0.834 (0.384-1.812)	0.647		

CI: Confidence intervaI; HR: hazard ratio; IMIG: International Mesothelioma Interest Group; p-Stage: pathological stage; p-S6RP: phosphorylated S6 ribosomal protein; p-MEK: phosphorylated mitogen-activated protein kinase kinase; p-mTOR: phosphorylated mammalian target of rapamycin; p-4EBP1: phosphorylated eukaryotic translation initiation factor 4E-binding protein 1; p-AKT: phosphorylated protein kinase B; p-ERK: phosphorylated extracellular signal-regulated kinase.

better in the p-mTOR-positive group than in the p-mTOR-negative group (26). In the current study, multimodal treatment with neoadjuvant chemotherapy (cisplatin and pemetrexed) was successful for treating early-stage MPM, consistent with the results of the previous study.

Based on the results of this study, we believe that mTOR inhibitors would contribute to the improvement of prognosis in the treatment of mesothelioma. However, mTOR inhibitors alone are ineffective, and it is desirable to use an mTOR inhibitor in combination with cisplatin/pemetrexed. In the current study, univariate and multivariate analyses revealed that p-mTOR expression was a prognostic factor, and the results suggest that the use of mTOR inhibitors is promising. Based on the same hypothesis, clinical trials of mTOR inhibitors were conducted for MPM. The SWOG S0722 trial evaluated the efficacy and safety of everolimus for patients with mesothelioma after chemotherapy with platinum-based regimens. The ORR was 2%; the median PFS was 2.8 months, and the median overall survival was 6.3 months (27). Because mTOR inhibitors alone are less effective against mesothelioma than other cancer types, combination therapy with chemotherapy is used. Sirolimus and cisplatin alone or in combination inhibited the growth of mesothelioma cell lines (28). Temsirolimus and cisplatin in combination were effective for mesothelioma, and temsirolimus induced apoptosis in cisplatin-resistant

mesothelioma cell lines (26). In a previous study, we showed that everolimus or selumetinib alone had significant antitumor activity, and the combination of everolimus and selumetinib enhanced the individual antitumor activity in MPM xenograft models (29). We believe that the combination of mTOR inhibitors with other drugs is desirable rather than using mTOR inhibitors alone.

During multimodal therapy for MPM, preoperative chemotherapy is initially administered and surgery is only performed for patients who respond to the treatment. In the present study, the significant differences observed p-mTOR expression suggest that molecular-targeted therapy can be used during multimodal therapy for MPM.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

SM and SH designed the study. AK wrote the main article text and prepared the Figure. AF, TN, AN, MH, TT and NK were involved with sample collection. SM performed the data analysis. TT, TN provided project supervision. All Authors approved the final article.

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