Relation between outcomes and expression of estrogen receptor-α phosphorylated at Ser^{167} in endometrioid endometrial cancer

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Endometrial cancer (EC) is the most common gynecological cancer and is thought to be estrogen-related. The estrogen receptor (ER) is a biological target for EC that has attracted considerable attention over the years. For many EC patients, particularly those with endometrial endometrioid cancer (EEC), which are tumors that express high levels of the ER, the observed response rates to hormonal agents such as progestins, antiestrogens, and aromatase inhibitors have not been satisfactory; indeed, many patients with advanced or refractory disease eventually develop resistance to this type of therapy and median survival is short at 7–12 months.1,2

Both ligand-dependent and ligand-independent activation of the ERα is modulated by receptor phosphorylation and receptor phosphorylation is enhanced by ligand binding.3 The major phosphorylation sites of ERα reside in the N-terminal domain at serines 104, 105, 118, and 167. Phosphorylation at Ser^{167} was shown to be important in receptor binding to DNA.3 In breast cancer, which is also an estrogen-related tumor, one mechanism by which resistance to hormone therapy develops is through phosphorylation of ERα at Ser^{167} (p-Ser^{167}-ERα), a modification that allows the receptor to function in an estrogen-independent manner.4 It has also been reported that two signaling pathways, mammalian target rapamycin (mTOR)/p70 S6 kinase 1 (S6K1) and MAPK/p90 ribosomal S6 kinase (RSK), coordinately regulate p-Ser^{167}-ERα and the development of resistance, which can serve as a prognostic marker for breast cancer.5,6 Previously published in vivo data suggest that Akt is phosphorylated leading to active p-Ser^{167}-ERα and resulting in activation of ERα-dependent pathways involved in EC pathogenesis.7 However, to the best of our knowledge, there are no published reports regarding the influence of p-Ser^{167}-ERα and mTOR/S6K1 and MAPK/RSK activity on outcomes in EEC patients.

The current study was designed to investigate correlations between p-Ser^{167}-ERα levels in EEC with clinicopathological features, disease outcomes, and levels of phosphorylated mTOR/S6K1 and phosphorylated MAPK/RSK (p-mTOR/p-S6K1 and p-MAPK/ p-p90RSK, respectively), as determined by examination of medical records and by immunohistochemical analysis.

Materials and Methods

Patients. The study group comprised 103 EEC patients who underwent total abdominal or radical hysterectomy plus bilateral salpingo-oophorectomy with or without lymphadenectomy during a 5-year period at the University of Fukui Hospital (Fukui, Japan) (Table 1). Clinicopathological characteristics and follow-up data were obtained from the subjects’ medical records. Staging, histology, and grading criteria were based on the 2009 International Federation of Gynecology and Obstetrics surgical staging classification. Definitive diagnosis was determined by postoperative histopathology and all specimens were evaluated by subsequent immunohistochemical analysis.
Patients with deep myometrial invasion, cervical involvement, special histology (such as undifferentiated adenocarcinoma), or lymph-node metastasis were treated with four to six rounds of postoperative adjuvant chemotherapy consisting of 180 mg/m² paclitaxel and carboplatin, according to Chatelut’s formula (area under the curve = 5 mg/mL/min). No patient was treated with hormone therapy, whether past or current. All patients were evaluated for disease recurrence for at least 2 years by annual physical examination and pap smear of the vaginal vault. In addition, diagnostic imaging (including ultrasonography, computed tomography, and/or MRI) was carried out every 3–6 month along with analysis of tumor markers. This study was approved by the institutional review board of the University of Fukui Hospital and written informed consent was obtained from all patients.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissue was immunohistochemically stained using the avidin–biotin–peroxidase complex technique with an LSAB kit (Dako, Glostrup, Denmark). Sections (2.5-μm thick) were dewaxed in xylene for 15 min three times, dehydrated in alcohol, and subjected to antigen retrieval in a pressure cooker for 15 min in 10 mM sodium citrate buffer (pH 6.0). After cooling, sections were washed three times in PBS (pH 7.2). Endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide for 5 min. Non-specific binding of primary antibodies was blocked by incubating sections with diluted (Dako Protein Block Serum-Free) for 10 min at room temperature. Samples were then incubated overnight with primary antibodies to the following proteins, diluted in PBS: ER<α> (Ser167) (p-Ser167-ER<α>) (rabbit polyclonal, 1:100; Abcam, Cambridge, UK); p-MAPK (Thr202/Thr204) (rabbit monoclonal, 1:300); p-p90RSK (Thr359/Ser363) (rabbit polyclonal, 1:250); p-mTOR (Ser2448) (rabbit monoclonal, 49F9, 1:50) and p70S6 (p-S6K1) (rabbit monoclonal, 49D7, 1:50) (all from Cell Signaling Technology, Beverly, MA, USA). After washing with PBS, sections were incubated for 10 min with diluted biotinylated goat anti-mouse immunoglobulins (Dako LSAB kit, Bottle 1) as the secondary antibody. After incubation with the avidin–biotin–peroxidase complex (Dako LSAB kit, Bottle 2) for 10 min and washing with PBS, the signal was visualized with substrate and 3,3′-diaminobenzidine in chromogen solution (Dako EnVision+ kit). Sections were then counterstained with Mayer’s acidic hematoxylin and washed multiple times in alcohol (70–100%). After xylene treatment, sections were covered until used.

Sections from human colon and breast cancers were used as positive controls and, for negative controls, incubation with

| Table 1. Clinicopathological features of 103 endometrioid endometrial cancers |
|---------------------------------|------------------|------------------|
| No. (n = 103) | % |
| Patient age, years |
| Median | 59.19 ± 11.1 | NA |
| Range | 38–92 | NA |
| Clinical stage |
| I | 71 | 69 |
| II | 12 | 12 |
| III | 13 | 12 |
| IV | 7 | 7 |
| Histological grade |
| Grade 1 | 64 | 62 |
| Grade 2 | 25 | 24 |
| Grade 3 and undifferentiated | 14 | 14 |
| Myometrial invasion |
| <50% | 78 | 64 |
| >50% | 25 | 36 |
| LVSI |
| Positive | 21 | 20 |
| Negative | 77 | 75 |
| Miss | 5 | 5 |
| LN metastases |
| Positive | 6 | 6 |
| Negative | 80 | 77 |
| Miss | 17 | 17 |
| Recurrence |
| No recurrence | 89 | 86 |
| Recurrence | 14 | 14 |

LN, lymph node; LVSI, lymphovascular space invasion; Miss, missing data; NA, not applicable.

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the primary antibody was omitted. The intensity and distribution of p-Ser167-ERα, p-MAPK, p-p90RSK, p-mTOR, and p-S6K1 staining was evaluated using a semiquantitative method (IRS score) as previously described,(8) and was calculated as follows:

\[
IRS = \sum SI \times PP,
\]

where SI is the optical stain intensity (graded 0, no; 1, weak; 2, moderate; 3, strong staining) and PP is the degree of positively stained cells (defined as 0, no staining; 1, <10%; 2, 11–50%; 3, 51–80%; 4, >81%). Those IRS scores greater than 6 (IRS ≥ 6) were defined as “positive”, and less than 5 (IRS 0–5) as “negative”. Immunostaining was scored by two independent observers (T. K. and A. S.) who are specialists in gynecological pathology. Discrepancies of more than two points in either optimal stain intensities or in the degree of positively stained cells were rare. However, if such discrepancies occurred, these slides were again evaluated by both observers. If the observers could not reach agreement on evaluation of immunostainings, these cases were excluded from this analysis. Ultimately, staining data for p-Ser167-ERα, p-MAPK, p-p90RSK, and p-mTOR were available for 103 patients and staining data for p-S6K1 were available for 98 patients.

Table 2. Relationships between molecular markers phosphorylated estrogen receptor α at Ser167 (p-Ser167-ERα), p-MAPK, p90 ribosomal S6 kinase (p-p90RSK), mammalian target of rapamycin (p-mTOR), and p70 S6 kinase 1 (p-S6K1) in endometrioid endometrial cancers (n = 103)

|               | p-Ser167-ERα (cytoplasm) | p-MAPK | p-p90RSK | p-mTOR (nucleus) | p-mTOR (cytoplasm) | p-S6K1 |
|---------------|--------------------------|--------|----------|------------------|--------------------|--------|
|               | N | P | P-value | N | P | P-value | N | P | P-value | N | P | P-value | N | P | P-value |
| p-Ser167-ERα (nuclear) |                |        |          |                |        |          |                |        |          |                |        |          |                |        |          |
| N  | 84 | 8 | 0.001* | 81 | 11 | 0.001* | 51 | 41 | 0.955 | 74 | 18 | 0.199 | 45 | 47 | 0.83  |
| P  | 6  | 5 |         | 5  | 6  |         | 6  | 5  |         | 7  | 4  |         | 5  | 6  |         |
| p-Ser167-ERα (cytoplasm) |                |        |          |                |        |          |                |        |          |                |        |          |                |        |          |
| N  | NA | NA |         | 80 | 10 | 0.001* | 51 | 39 | 0.476 | 73 | 17 | 0.107 | 47 | 43 | 0.05  |
| P  | NA | NA |         | 6  | 7  |         | 6  | 7  |         | 8  | 5  |         | 3  | 10 |         |
| p-MAPK |                |        |          |                |        |          |                |        |          |                |        |          |                |        |          |
| N  | NA | NA |         | NA | 38 | 0.828  | 69 | 17 | 0.375 | 44 | 42 | 0.23  | 37 | 44 | 0.092 |
| P  | NA | NA |         | 9  | 8  |         | 12 | 5  |         | 6  | 11 |         | 4  | 13 |         |
| p-p90RSK |                |        |          |                |        |          |                |        |          |                |        |          |                |        |          |
| N  | NA | NA |         | NA | NA |         | 46 | 11 | 0.57  | 33 | 24 | 0.04* | 30 | 24 | 0.002* |
| P  | NA | NA |         | NA | NA |         | 35 | 11 |         | 17 | 29 |         | 11 | 33 |         |
| p-mTOR (nucleus) |                |        |          |                |        |          |                |        |          |                |        |          |                |        |          |
| N  | NA | NA |         | NA | NA |         | NA | NA |         | NA | NA |         | 17 | 24 | 0.207 |
| P  | NA | NA |         | NA | NA |         | NA | NA |         | NA | NA |         | 31 | 26 |         |

P-values from χ²-tests. *P < 0.05. N, negative; P, positive; NA, not applicable.

Table 3. Relationships between the molecular markers phosphorylated estrogen receptor α at Ser167 (p-Ser167-ERα), p-MAPK, p90 ribosomal S6 kinase (p-p90RSK), mammalian target of rapamycin (p-mTOR), and p70 S6 kinase 1 (p-S6K1) and clinicopathological factors in endometrioid endometrial cancers (n = 103)

|               | p-Ser167-ERα (nuclear) | p-Ser167-ERα (cytoplasm) | p-MAPK | p-p90RSK | p-mTOR (nucleus) | p-mTOR (cytoplasm) | p-S6K1 |
|---------------|--------------------------|--------------------------|--------|----------|------------------|--------------------|--------|
|               | N | P | P-value | N | P | P-value | N | P | P-value | N | P | P-value | N | P | P-value |
| Stage |                |        |          |                |        |          |                |        |          |                |        |          |                |        |          |
| I–II | 79 | 7 | 0.06  | 76 | 10 | 0.495 | 71 | 15 | 0.565 | 46 | 40 | 0.395 | 69 | 17 | 0.375 |
| III–IV | 13 | 4 | 0.122 | 14 | 3  |         | 15 | 2  |         | 11 | 6  |         | 12 | 5  |         |
| Grade |                |        |          |                |        |          |                |        |          |                |        |          |                |        |          |
| I–II | 82 | 8 | 0.122 | 80 | 10 | 0.225 | 75 | 15 | 0.907 | 45 | 45 | 0.004* | 75 | 15 | 0.002* |
| III | 10 | 3  |         | 10 | 3  |         | 11 | 2  |         | 12 | 1  |         | 6  | 7  |         |
| Invasion |                |        |          |                |        |          |                |        |          |                |        |          |                |        |          |
| >50% | 70 | 8 | 0.806 | 68 | 10 | 0.914 | 64 | 14 | 0.484 | 40 | 38 | 0.143 | 65 | 13 | 0.4  |
| <50% | 22 | 3  |         | 23 | 3  |         | 17 | 8  |         | 6  | 7  |         | 6  | 7  |         |
| LVSI |                |        |          |                |        |          |                |        |          |                |        |          |                |        |          |
| N | 70 | 7 | 0.735 | 68 | 9  | 0.993 | 63 | 14 | 0.210 | 39 | 38 | 0.017* | 63 | 14 | 0.297 |
| P | 15 | 2  |         | 15 | 2  |         | 16 | 1  |         | 14 | 3  |         | 12 | 5  |         |
| Recurrence |                |        |          |                |        |          |                |        |          |                |        |          |                |        |          |
| N | 83 | 6 | 0.001* | 80 | 9  | 0.053 | 75 | 14 | 0.593 | 48 | 41 | 0.469 | 74 | 15 | 0.005* |
| P | 9  | 5  |         | 10 | 4  |         | 11 | 3  |         | 9  | 5  |         | 7  | 7  |         |

P-values from χ²-tests. *P < 0.05. LVSI, lymphovascular space invasion; N, negative; P, positive.
results

Phosphorylated Ser\textsuperscript{167}ER\textsubscript{α} was observed in the nuclei and cytoplasm of EEC cells (in 10.7% and 12.6% of the cells, respectively) (Fig. 1), as was p-mTOR (in 21.4% and 51.5% of the cells, respectively). Phosphorylated MAPK, p-p90RSK, and p-S6K1 were observed only in the cytoplasm (in 16.5%, 44.7%, and 55.3% of the cells, respectively).

The prognostic relevance of levels of p-Ser\textsuperscript{167}ER\textsubscript{α}, mTOR, p-MAPK, p-p90RSK, and p-S6K1 was analyzed using a multivariate proportional hazards model adjusted for established clinical prognostic factors; depth of tumor invasion, LVSI, histological grade, and stage (Table 4). Histological grade and LVSI were independent prognostic factors for RFS (hazard ratio [HR] = 38.285; 95% confidence interval [CI], 1.882–778.7; P = 0.018; and HR = 6.567; 95% CI, 1.087–39.676; P = 0.040, respectively), but nuclear p-Ser\textsuperscript{167}ER\textsubscript{α} level was not independent (HR = 6.707; 95% CI, 0.419–107.406; P = 0.179). In addition, there were no correlations between nuclear p-mTOR and recurrence site.
Table 4. Prognostic factors for relapse-free survival in endometrioid endometrial cancers (n = 103): Multivariate Cox proportional-hazards regression model analysis

| Factor                      | HR     | 95% CI    | P-value |
|-----------------------------|--------|-----------|---------|
| Stage                       | 0.566  | 0.065     | 4.958   | 0.607   |
| Grade                       | 38.285 | 1.882     | 778.700 | 0.018*  |
| Invasion                    | 1.650  | 0.146     | 18.637  | 0.686   |
| LVSI                        | 6.567  | 1.087     | 39.676  | 0.040*  |
| p-mTOR (nucleus)            | 2.220  | 0.445     | 11.071  | 0.331   |
| p-mTOR (cytoplasm)          | 1.592  | 0.346     | 7.322   | 0.550   |
| p-Ser167-ERα (nuclear)      | 6.707  | 0.419     | 107.406 | 0.179   |
| p-Ser167-ERα (cytoplasm)    | 1.464  | 0.105     | 20.386  | 0.777   |
| p-S6K1                      | 0.265  | 0.037     | 1.905   | 0.187   |
| p-MAPK                      | 1.142  | 0.180     | 7.249   | 0.888   |
| p-p90RSK                    | 5.882  | 0.533     | 62.595  | 0.142   |

*P < 0.05. CI, confidence interval; HR, hazards ratio; LVSI, lymphovascular space invasion; p-mTOR, phosphorylated mammalian target of rapamycin; p-p90RSK, phosphorylated p90 ribosomal S6 kinase; p-S6K1, phosphorylated p70 S6 kinase 1; p-Ser167-ERα, phosphorylated estrogen receptor α at Ser167.

Discussion

In this study we identified that, in EEC, nuclear p-Ser167-ERα is the result of cooperation between mTOR/S6K1 and MAPK/RSK signaling pathways, and indicates development of advanced disease carrying a poor prognosis. Therefore, increased nuclear p-Ser167-ERα may play a pivotal role in the neoplastic process, and may be a marker indicating poor prognosis; this is the opposite of the situation seen in breast cancer.

Although p-AKT was not associated with disease-free survival, p-AKT positivity was associated with a reduction in overall survival. They suggested that p-P-p90RSK and p-MAPK, rather than p-AKT, may mediate the phosphorylation of p-Ser167-ERα in breast cancer cells, and that p-AKT is instead involved in regulating other cellular processes that lead to reduced patient survival. The statement that p-AKT can potentially induce the phosphorylation of p-Ser167-ERα appears to contradict the earlier statement that the authors of the study suggested that it was p-p90RSK and p-MAPK, rather than p-AKT, that may mediate the phosphorylation of p-Ser167-ERα in breast cancer cells.

To our knowledge, there have only been a few reports of studies in which the relationship between p-ERα Ser167 and EEC have been explored. Vilgelm et al. (7) indicated that the loss of phosphatase and tensin homologue deleted on chromosome ten (PTEN) and AKT activation results in a Ser167 phosphorylation-dependent enhancement of ERα transcriptional activity that is independent of estrogen and plays a pivotal role in the neoplastic process. Shah et al. (3) identified a connection between the ER coactivators of steroid receptor coactivator (Src) kinase and p-ERα Ser167 through activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which, in turn, potentiates tamoxifen agonist action. These data supported the idea that Ser167 phosphorylation of the ER through activation of the PI3K/AKT pathway in the endometrium is an important process.

Recent molecular profiling has shown that increased PI3K/AKT/mTOR signaling is associated with aggressive disease and poor prognosis, irrespective of endometrial cancer tumor. A major downstream effector of AKT is mTOR complex1 (mTORC1); its downstream targets, such as ribosomal S6K1, control protein synthesis. Another mTOR complex, mTORC2, participates in the activation of AKT. In our previous study of a series of 82 patients with ECC, we reported that nuclear mTORC1 was significantly elevated in poorly differentiated tumors with lymph node involvement and in patients with shorter survival. In the present study, immunohistochemical evaluation of the expression of p-S6K1 indicated that it was positively correlated with LVSI. Lymphovascular space invasion includes lymphatic vessel invasion and blood vessel invasion, thought to be the beginnings of lymphogenous and hematogenous metastases, respectively. Koskas et al. (12) suggested that LVSI should be considered as an independent risk factor for lymph node metastasis. It is therefore a reasonable finding that overexpression of p-S6K1 was observed in the present LVSI cases.

Signaling of mTORC1 is also involved in cross-talk with MAPK signaling. The corresponding effectors of these pathways, S6K1 and RSK respectively, have been shown to converge on a common set of targets, most notably in control of protein translation. In this study, we identified nuclear p-Ser167-ERα as a recipient of coordinated phosphorylation inputs from MAPK and mTOR and showed that nuclear p-Ser167-ERα might be related to the biological behavior of ECC. These findings are similar to results reported in breast cancer; Yamnik et al. (5,6) reported that mTOR/S6K1 and MAPK/RSK coordinately regulate p-Ser167-ERα and the development of resistance, which can serve as a prognostic marker for breast cancer.

In conclusion, we showed that, in EEC, nuclear p-Ser167-ERα was strongly positively correlated with p-ERα and p-S6K1. The coordinate action of mTOR/S6K1 and MAPK/RSK pathways provide a strong stimulus for EEC tumor growth, and may contribute to the development of advanced stages of cancer with poor prognosis. We suggest that dual inhibition of the mTOR/S6K1 and MAPK/RSK signaling pathways, which lead to ERα activation and stimulation of ECC development, may result in better clinical responses in advanced EEC patients.

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Disclosure Statement

The authors have no conflict of interest.
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