Phospho-Serine-118 Estrogen Receptor-α Expression Is Associated with Better Disease Outcome in Women Treated with Tamoxifen

Leigh C. Murphy,1 Yulian Niu,2 Linda Snell,2 and Peter Watson2
1Manitoba Institute of Cell Biology, Department of Biochemistry and Medical Genetics and 2Department of Pathology, University of Manitoba, Winnipeg, Manitoba, Canada

ABSTRACT

Purpose: The purpose of this research was to determine whether estrogen receptor α specifically phosphorylated at Ser118 is associated with clinical outcome in primary breast tumors from estrogen receptor-positive and node-negative breast cancer patients.

Experimental Design: Estrogen receptor α specifically phosphorylated at Ser118 was determined by immunohistochemistry in 117 primary breast tumors from node-negative patients who were subsequently treated with adjuvant tamoxifen. The relationship of estrogen receptor α specifically phosphorylated at Ser118 expression to disease-free survival and overall survival was determined.

Results: Estrogen receptor α specifically phosphorylated at Ser118 was limited to estrogen receptor α ligand binding assay-positive tumors and among this subset was expressed in 70 (62%) of these tumors. Estrogen receptor α specifically phosphorylated at Ser118 expression was more frequently observed in progesterone receptor-positive tumors compared with progesterone receptor-negative tumors (χ² test, P = 0.012, n = 113). A significant correlation was also seen between estrogen receptor α specifically phosphorylated at Ser118 and progesterone receptor levels (Spearman r = 0.236, P = 0.0118, n = 113). Kaplan-Meier outcome analysis showed that patients whose primary tumors expressed estrogen receptor α specifically phosphorylated at Ser118 had a longer disease-free survival (P = 0.0018, n = 113) and a trend toward better overall survival, but this was not statistically significant. Among the subset of progesterone receptor-positive tumors, progesterone receptor-positive/estrogen receptor α specifically phosphorylated at Ser118-positive patients had a significantly longer disease-free survival that progesterone receptor-positive/estrogen receptor α specifically phosphorylated at Ser118-negative patients (P = 0.0041).

Conclusions: Our data suggest that estrogen receptor α specifically phosphorylated at Ser118 is a marker of a functional, intact ligand-dependent estrogen receptor signaling pathway in breast cancer and that estrogen receptor α specifically phosphorylated at Ser118 status has the potential to provide a more precise biomarker of responsiveness to endocrine therapy in conjunction with estrogen receptor α and progesterone receptor status.

INTRODUCTION

Tumor expression of estrogen receptor-α is the gold-standard biomarker for predicting responsiveness of breast cancer to endocrine therapies such as the antiestrogen tamoxifen (1). This is basically due to estrogen receptors central role within the estrogen signaling pathway (2) such that when tamoxifen binds to estrogen receptor a conformational change occurs resulting in the inhibition of estrogen signaling and consequently the proliferative action of estrogen on estrogen receptor-positive human breast cancer cells (3). However, despite the success of tamoxifen therapy (4), some primary estrogen receptor α-positive tumors do not respond to tamoxifen (de novo resistance), and many that originally respond eventually acquire tamoxifen resistance, despite continued expression of estrogen receptor (5). Therefore, estrogen receptor is not a perfect biomarker for the prediction of endocrine therapy responsiveness. The identification of more precise markers of treatment response would be useful in the treatment of breast cancer patients.

There are several different phosphorylation sites on estrogen receptor α that may modulate estrogen receptor action, although the exact functional role in vivo of phosphorylation at the various sites is unclear (6). Phosphorylation of estrogen receptor α at Ser118 is being investigated extensively and may have a role in the mechanism by which estrogen receptor α is transcriptionally activated by both estrogen and other cross-talk pathways, in particular the mitogen-activated protein kinase (MAPK) pathway (6, 7). We validated previously an antibody for immunohistochemistry in paraffin-embedded and formalin-fixed sections of human breast tumors, which specifically detects estrogen receptor α phosphorylated at Ser118 (8). Surprisingly our preliminary data suggested that estrogen receptor α specifically phosphorylated at Ser118 was associated with a more differentiated phenotype and other markers of good prognosis in human breast cancer. This led us to speculate that estrogen receptor α specifically phosphorylated at Ser118 may more accurately reflect an intact ligand-dependent estrogen receptor signal transduction pathway within a breast tumor and increased likelihood of response to endocrine therapy. In the current study we examined this hypothesis by determining the

Received 1/30/04; revised 4/7/04; accepted 4/22/04.

Grant support: Canadian Institutes for Health Research (CIHR) and the Canadian Foundation for Innovation (CFI).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: P. Watson is a Canadian Institutes for Health Research/Medical Research Council Scientist.

Requests for reprints: Leigh Murphy, Manitoba Institute of Cell Biology, 675 McDermot Avenue, University of Manitoba, Winnipeg, Manitoba, Canada, R3E 0V9. Phone: 204-787-4071; Fax: 204-787-2190; E-mail: lcmurphy@cc.umanitoba.ca.

©2004 American Association for Cancer Research.
relationship of estrogen receptor α specifically phosphorylated at Ser\(^{118}\) to disease outcome in a cohort of estrogen receptor-positive, node-negative breast cancer patients who were treated with tamoxifen.

**MATERIALS AND METHODS**

**Human Breast Tumors.** All of the cases used for this study were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As described previously (9), tissues are accrued to the bank from cases at multiple centers within Manitoba, rapidly collected, and processed to create matched formalin-fixed, embedded, and frozen tissue blocks for each case with the mirror image surfaces oriented by colored inks. The histology of every sample in the bank is uniformly interpreted by a pathologist in hematoxylin and eosin-stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin blocks from cases for this study. For each case interpretation included an estimate of the cellular composition (including the percentage of invasive epithelial tumor cells and stroma), tumor type, and tumor grade (Nottingham score). Clinical steroid receptor status was determined for all of the cases by ligand binding assay performed on an adjacent portion of tumor tissue. Tumors with estrogen receptor levels ≥3 fmol/mg of total protein were considered estrogen receptor positive.

**Clinical and Pathological Characteristics of the Patient Cohort.** The selection and characteristics of the cohort used for this study have been described (9). Briefly, 140 invasive breast carcinomas were selected from the bank. All of the cases were axillary lymph node negative and had been treated with surgery with or without radiation and then tamoxifen therapy. The majority of these tumors were estrogen receptor-positive, although some (10%) were estrogen receptor-negative tumors from older patients with otherwise good prognostic markers that were nevertheless treated with adjuvant tamoxifen therapy. The clinicopathological characteristics of the final patient cohort are shown in Table 1. Criteria for the determination of variables are: (1) estrogen receptor positivity ≥3 fmol/mg protein and progesterone receptor positivity >15 fmol/mg protein; (2) grade determined by the Nottingham system was assigned as low (scores 3–5), moderate (scores 6–7), or high (scores 8–9); (3) tumor size was categorized as small (≤2 cm) or large (>2 cm); and (4) tumor inflammation was assessed in the tumor tissue section and increasing degrees of inflammation scored using a subjective scale from 1 to 5.

**Immunohistochemistry.** Immunohistochemistry was performed on serial 5-μm sections from a representative, formalin-fixed, paraffin-embedded archival tissue block from each tumor. In all of the cases, tissue samples were fixed for 18–24 h in 10% buffered formalin before routine embedding in paraffin wax. Five micrometer sections were cut, mounted on Superfrost/Plus slides (Fisherbrand), and dried overnight at 37°C. Immunohistochemistry for estrogen receptor α specifically phosphorylated at Ser\(^{118}\) used mouse monoclonal antibodies specific for estrogen receptor α phosphorylated on serine 118 (#2511, 16J Cell Signaling Technology, NEB Ltd., Mississauga, Ontario, Canada). Antibodies were applied using an automated tissue immunostainer (Discovery Module, Ventana Medical Systems, Phoenix, AZ), and 3,3′-diaminobenzidine immunohistochemistry kit and bulk reagents were supplied by the manufacturer. Briefly, the Discovery staining protocol was set to “Standard Cell Conditioning,” followed by 12-hour incubation with primary antibody and 3-minute incubation with secondary antibody. Primary antibody concentrations initially applied to the Ventana were 1:200 for estrogen receptor α specifically phosphorylated at Ser\(^{118}\) translating into final concentrations of 1:600 after 1:3 dilution with buffer dispensed onto the slide with the primary antibody. The slides are then counterstained using hematoxylin followed by a Bluing Reagent resulting in pale blue nuclei. After rinsing, dehydrating (graded alcohols), and clearing (xylol), the glass coverslips were applied using Permount. As described previously (8), levels of nuclear estrogen receptor α specifically phosphorylated at Ser\(^{118}\) expression were scored semi-quantitatively under the light microscope. Scores were obtained by estimating average signal intensity (scale of 0–3) and the proportion of epithelial cells showing a positive signal (0–100%). The intensity and proportion scores were then multiplied to give an overall immunohistochemistry score.

**Statistical Analysis.** Correlations of estrogen receptor α specifically phosphorylated at Ser\(^{118}\) immunohistochemistry scores with clinical pathological variables were assessed using the Spearman test. Differences between tumor subgroups were tested using the Mann-Whitney rank sum test, two-sided. Relapse-free survival was defined as the time from initial surgery to the date of clinically documented local or distant disease recurrence or death attributed to breast cancer. Overall survival was defined as the time from initial surgery to the date of death attributable to breast cancer. The association with relapse and survival was assessed by univariate analysis (log-rank test and Kaplan-Meier method). All of the tests were performed using Prism (GraphPad Inc., San Diego, CA) statistical analysis software.

**RESULTS**

Tissue blocks from all of the 140 cases in the cohort were sectioned and underwent immunohistochemistry for the detection of estrogen receptor α specifically phosphorylated at Ser\(^{118}\) using the antibody validated previously (8). However, some

---

**Table 1** Clinicopathological characteristics of the study cohort

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−ve</td>
<td>4</td>
<td>3.4</td>
</tr>
<tr>
<td>+ve</td>
<td>113</td>
<td>96.6</td>
</tr>
<tr>
<td>Progesterone receptor &gt;15 fmol/mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>82</td>
<td>70</td>
</tr>
<tr>
<td>−ve</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>41</td>
<td>35.1</td>
</tr>
<tr>
<td>Int</td>
<td>59</td>
<td>50.4</td>
</tr>
<tr>
<td>High</td>
<td>17</td>
<td>14.5</td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 2 cm</td>
<td>41</td>
<td>35</td>
</tr>
<tr>
<td>≥ 2 cm</td>
<td>76</td>
<td>65</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 50</td>
<td>15</td>
<td>12.8</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>102</td>
<td>87.2</td>
</tr>
</tbody>
</table>
blocks had been partially exhausted, and intact sections containing sufficient tumor cells for analysis were obtained for 117 tumors, and only these have been included in this analysis. One hundred and thirteen (97%) of tumors were estrogen receptor positive and 4 were estrogen receptor negative as determined previously by ligand binding assay (no estrogen receptor α specifically phosphorylated at Ser118 expression was detected in any of these estrogen receptor ligand binding-negative tumors). Of the 113 estrogen receptor-positive tumors, 70 (62%) had detectable nuclear staining for estrogen receptor α specifically phosphorylated at Ser118.

Analysis of correlations of estrogen receptor α specifically phosphorylated at Ser118 immunochemistry scores with several variables was done using Spearman tests, initially using the entire 117 cases. A significant positive correlation was found between P-Ser118 estrogen receptor α expression and progesterone receptor levels as determined by ligand binding analysis (Spearman r = 0.273, P = 0.0029, n = 117). Consistent with this correlation, the median level of progesterone receptor in estrogen receptor α specially phosphorylated at Ser118-positive tumors was significantly higher than that in estrogen receptor α specifically phosphorylated at Ser118-negative tumors (42.5 versus 16.1 fmol/mg protein, Mann Whitney two-tailed P = 0.002, n = 117). The correlation analysis was also done after eliminating the 4 estrogen receptor-negative tumors. A significant correlation remained between estrogen receptor α specifically phosphorylated at Ser118 and progesterone receptor levels (Spearman r = 0.236, P = 0.0118, n = 113), and the median level of progesterone receptor in the estrogen receptor α specifically phosphorylated at Ser118-positive tumors was significantly higher than that in the estrogen receptor α specifically phosphorylated at Ser118-negative tumors (42.5 versus 20 fmol/mg protein, Mann-Whitney two tailed P = 0.0093, n = 113). In addition, in categorical analysis where progesterone receptor positivity was defined as >15 fmol/mg of protein, estrogen receptor α specifically phosphorylated at Ser118 expression was more frequently observed on progesterone receptor-positive tumors (χ², two-sided, P = 0.012, n = 113). There was no statistically significant difference in total estrogen receptor α levels between estrogen receptor α specifically phosphorylated at Ser118-positive and -negative tumors (mean estrogen receptor α, 46 versus 54 fmol/mg protein, P = 0.495, n = 113).

In addition a weak inverse correlation of estrogen receptor α specifically phosphorylated at Ser118 expression and tumor grade was found that showed borderline significance (Spearman r = −0.1819, P = 0.0497, n = 117) in all of the tumors and in the estrogen receptor ligand-binding assay-positive subgroup (r = −0.177, P = 0.06, n = 113).

To determine whether estrogen receptor α specifically phosphorylated at Ser118 status was related to clinical outcome in patients treated with tamoxifen, the relationship of estrogen receptor α specifically phosphorylated at Ser118 expression determined as a binary factor (positive, any detectable nuclear staining versus negative, no detectable nuclear staining) to clinical outcome was investigated. In many cases expression was quite focal within the tumor. However, in keeping with previous studies to determine the clinical significance of estrogen receptor α expression by immunohistochemistry (10), weak positive staining in >1% of tumor cells was regarded as positive estrogen receptor α specifically phosphorylated at Ser118.

Disease-free survival and overall survival Kaplan-Meier plots are shown in Fig. 1 for the cohort without the 4 estrogen receptor α-negative patients (however, their inclusion in the analysis did not significantly alter the results obtained). Those patients whose primary tumors expressed estrogen receptor α specifically phosphorylated at Ser118 had a longer disease-free survival than those whose tumors were estrogen receptor α specifically phosphorylated at Ser118 negative (Fig. 1A, P = 0.0018, n = 113). Although there is an apparent trend with regard to overall survival (Fig. 1B), this was not statistically significant, likely due to the small number of events (deaths) occurring in this generally good prognosis (estrogen receptor α positive, node negative) cohort of breast cancer patients. A similar analysis was carried out using progesterone receptor status (progesterone receptor positivity defined as >15 fmol/mg protein), and those patients with progesterone receptor-positive tumors demonstrated both significantly decreased recurrence (Fig. 1C, P = 0.0079, n = 113) and significantly increased survival (Fig. 1D, P < 0.0001). To determine whether estrogen receptor α specifically phosphorylated at Ser118 status together with progesterone receptor status could potentially be an even better prediction of disease outcome we compared time to recurrence and overall survival between progesterone receptor-positive/estrogen receptor α specifically phosphorylated at Ser118-positive patients to progesterone receptor-positive/estrogen receptor α specifically phosphorylated at Ser118-negative patients. Progesterone receptor-positive/estrogen receptor α specifically phosphorylated at Ser118-positive patients had a significantly longer disease-free survival than progesterone receptor-positive/estrogen receptor α specifically phosphorylated at Ser118-negative patients (Fig. 1E, P = 0.0041, n = 81) treated with tamoxifen. No significant differences in overall survival were seen, possibly due to the small number of events (death) that occurred in this very good prognostic group. No significant association of grade or size with clinical outcome was found.

**DISCUSSION**

We had undertaken previously a study in a small cohort of primary human breast cancers to validate and explore the expression of estrogen receptor α specifically phosphorylated on Ser118 (8). This study provided evidence for the reliable detection of estrogen receptor α specifically phosphorylated at Ser118 in multiple samples of human breast biopsies. This study also suggested that estrogen receptor α specifically phosphorylated at Ser118 detection is associated with a more differentiated phenotype and other markers of good prognosis in breast cancer and may be a good marker of an intact estrogen signaling pathway and, therefore, responsiveness to endocrine therapies. The current study addresses the association of estrogen receptor α specifically phosphorylated at Ser118 expression and clinical outcome in a subset of breast cancer patients, with generally good prognosis (node negative and estrogen receptor positive), who were treated with tamoxifen. The results suggest that the detection of estrogen receptor α specifically phosphorylated at Ser118 is significantly associated with a better clinical outcome in this cohort. Previously in similar cohorts progesterone recep-
tor expression was associated with clinical outcome in patients treated with tamoxifen (11), and our data are consistent with these previous data and suggest that both progesterone receptor and estrogen receptor α specifically phosphorylated at Ser118 are indicators of outcome. In this small cohort the established progesterone receptor marker appeared better, but our data showing a significant increase in disease-free survival in progesterone receptor-positive/estrogen receptor α specifically phosphorylated at Ser118-positive compared with progesterone receptor-positive/estrogen receptor α specifically phosphorylated at Ser118-negative patients suggest that the combination of these two biomarkers has the potential to provide more accurate prediction of disease outcome in patients treated with tamoxifen. This suggests that estrogen receptor α specifically phosphorylated at Ser118 status can provide extra prognostic and treatment response information over and above total estrogen receptor α and progesterone receptor status. Additional studies are warranted to determine the full significance of this observation.

Our data also demonstrate a positive relationship between progesterone receptor levels and progesterone receptor status with estrogen receptor α specifically phosphorylated at Ser118, suggesting that both progesterone receptor and estrogen receptor α specifically phosphorylated at Ser118 reflect an intact, functional, estrogen receptor signaling pathway and a possible regulatory relationship of estrogen receptor α specifically phosphorylated at Ser118 and progesterone receptor expression. Our previous study found no relationship of estrogen receptor α specifically phosphorylated at Ser118 status and progesterone receptor status. It did, however, find a positive correlation of estrogen receptor α specifically phosphorylated at Ser118 with estrogen receptor status as one might expect. These apparent differences can be explained by the nature of the two cohorts used in these studies. The initial cohort was designed to be a scanning tool to validate an assay and to broadly identify possible correlations with known prognostic markers. As such the initial cohort was much smaller and composed of equivalent numbers of estrogen receptor α-positive and -negative tumors, in contrast to the current cohort, which is composed of only estrogen receptor α-positive tumors.

Both the current and previous results support the conclusion that detection of estrogen receptor α specifically phosphorylated at Ser118 is a marker of a functional, ligand-dependent estrogen receptor signaling pathway. This was somewhat surprising given the association of growth factor pathway-mediated phosphorylation of Ser118 of estrogen receptor α with ligand-independent activation of the receptor and the speculation that...
this could be a mechanism associated with estrogen independence and/or tamoxifen resistance in human breast cancer, especially because several studies have correlated amplification and overexpression of HER-2 and epidermal growth factor receptor with resistance to endocrine therapy in breast cancer in vivo (12, 13). Our results suggest that increased expression of estrogen receptor α specifically phosphorylated at Ser118 is not associated with de novo tamoxifen resistance; however, we cannot exclude the possibility that estrogen receptor α specifically phosphorylated at Ser118 expression can be additionally up-regulated and associated with acquired tamoxifen resistance.

If estrogen receptor α specifically phosphorylated at Ser118 is a marker of a functional estrogen receptor α signaling pathway, what does this imply mechanistically? The role of phosphorylation of Ser118 on estrogen receptor α is unknown. Site-directed mutagenesis studies involving Ser118 and estrogen receptor transcriptional activity have given widely varying results (6) and may be influenced by different cellular backgrounds as well as different types of promoters used in each study (6). Ligand and DNA binding are not affected by phosphorylation of Ser118; however, effects on interaction with non-classical promoters, protein turnover, and so forth, have not been examined. It is known that stimulation of breast cancer cells with both 17β-estradiol and/or growth factors can result in phosphorylation of estrogen receptor α on Ser118 (14). Phosphorylation of Ser118 due to 17β-estradiol, however, may be mediated at least in part by cyclin-dependent kinase 7 in transiently transfected COS-1 cells (15) and possibly MCF7 cells (14) and in both cases is independent of MAPK. The growth factor-mediated phosphorylation of Ser118 is ligand independent and either directly or indirectly mediated by MAPK (7, 14). Active MAPK is, however, significantly up-regulated in many breast tumors compared with matched normal breast tissue, as well as during progression of breast cancer (16, 17) suggesting the possibility that it could be involved in the ligand-independent activation of estrogen receptor α during progression and be associated with endocrine resistance. Indeed, we identified previously a correlation in primary breast tumors between estrogen receptor α specifically phosphorylated at Ser118 and active MAPK suggesting that active MAPK could be directly or indirectly involved in the phosphorylation (7, 14). However, our correlative data and the current clinical outcome data suggest that even if this is correct, it does not result in a poor outcome in those patients treated with tamoxifen therapy (16) and is unlikely a mechanism of de novo tamoxifen resistance. This does not exclude the MAPK pathway being involved in acquired tamoxifen resistance.

In conclusion, our data support the association of estrogen receptor α specifically phosphorylated at Ser118 in primary breast tumors and a good clinical outcome in estrogen receptor α-positive, node-negative patients treated with tamoxifen. This suggests that estrogen receptor α specifically phosphorylated at Ser118 is a marker of a functional, ligand-dependent estrogen receptor signaling pathway in breast cancer and that estrogen receptor α specifically phosphorylated at Ser118 determination together with progesterone receptor status may provide a better indicator of response to endocrine therapy than total estrogen receptor α status alone.

REFERENCES


Phospho-Serine-118 Estrogen Receptor-α Expression Is Associated with Better Disease Outcome in Women Treated with Tamoxifen

Leigh C. Murphy, Yulian Niu, Linda Snell, et al.


Updated version Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/10/17/5902

Cited articles This article cites 16 articles, 9 of which you can access for free at: http://clincancerres.aacrjournals.org/content/10/17/5902.full#ref-list-1

Citing articles This article has been cited by 16 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/10/17/5902.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.