Src Kinase and Mitogen-Activated Protein Kinases in the Progression from Normal to Malignant Endometrium

Mohamed Mokhtar Desouki and Brian G. Rowan  
Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio

ABSTRACT

Purpose: The purpose of this research was to determine whether a correlation exists between the levels of activated mitogen-activated protein kinase (MAPK) and Src kinases and the progression from normal to malignant endometrium.

Experimental Design: We measured total and phosphorylated levels for extracellular signal-regulated kinase 1/2, p38, stress-activated protein kinase/c-Jun NH2-terminal kinase, and Src kinases from 33 frozen endometrial adenocarcinomas and 38 benign endometrial specimens by quantification of signals from Western blots using antibodies against these kinases.

Results: Elevated phospho-extracellular signal-regulated kinase 1/2 (150 ± 40 versus 46 ± 7; P = 0.03), phospho-Src (28 ± 5 versus 4 ± 1), and phospho-p38 (131 ± 16 versus 27 ± 7; P < 0.001) was detected in benign versus malignant endometrium when the Western blot signal of activated kinase was normalized to total kinase levels and β actin. A modest increase in active c-Jun NH2-terminal kinase was detected in carcinoma versus benign specimens (51 ± 13 versus 43 ± 10; P = 0.8). Expression of total kinases (normalized to β-actin) was higher in carcinoma versus benign specimens, respectively (extracellular signal-regulated kinase 1/2, 9 ± 2 versus 0.7 ± 0.1; Src, 7 ± 2 versus 0.4 ± 0.1; stress-activated protein kinase c-Jun NH2-terminal kinase, 2 ± 0.4 versus 0.2 ± 0.02; P < 0.001; and p38, 1 ± 0.2 versus 0.4 ± 0.1; P < 0.01). Immunohistochemistry for active and total Src kinases and MAPKs detected positive staining in epithelial and stroma cells.

Conclusions: These data demonstrated that, in contrast with breast cancer, the progression from normal to malignant endometrium is not associated with activation of MAPK and Src kinases. Elevation of these active kinases in benign endometrium may contribute to endometrial resistance to the antiestrogen action of tamoxifen.

INTRODUCTION

Phosphorylated members of the mitogen activated protein kinase (MAPK) family [MAPKs, extracellular signal-regulated kinases (ERKs) 1 and 2 (P44/42), p38 and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK)] and Src kinase play an important role in the regulation of cell growth and differentiation (1). For several tumor types there is a correlation between the degree of kinase activation and the progression to malignancy, which may be exploited for therapeutic intervention with agents that target the kinases. (reviewed in Ref. 2 and references therein).

In breast cancer, the growth of most early stage tumors is estrogen-dependent and influenced by the degree of activation of kinase pathways. There is a well-established cross-talk among estrogen, tamoxifen, and growth factor signaling pathways in breast cancer. Estrogen and tamoxifen up-regulate growth factors and growth factor receptors and can activate certain signal transduction pathways (3–10). Alternately, activation of cellular signaling pathways can potentiate steroid receptor activation and reproduce estrogen effects on proliferation and gene transcription. Seminal in vivo experiments established that the proliferative effect of epidermal growth factor on the uterus required functional estrogen receptor (ER) as established using the ERα knockout mouse model (11). In cell culture studies, elevation of intracellular cyclic AMP can enhance steroid-dependent activation of the ER as well as induce a ligand-independent activation of the ER (12), and chicken progesterone receptor (13). In breast cancer cell lines, cross-talk exists between MAPK and progesterone receptors that results in a decrease in the biological effects of progesterone (14).

Significantly elevated MAPK activity (ERK1/2) has been detected in invasive breast carcinomas compared with surrounding benign breast tissue (15). Furthermore, elevated Src kinase activity was detected in the progression of breast cancers with the acquisition of steroid independence and may indicate that growth of late-stage tumors is regulated by peptide growth factors that stimulate Src kinases and MAPKs (16).

Steroid-dependent tumors may use MAPK and Src kinase pathways in several ways to facilitate tumor growth. First, ER phosphorylation by these kinase pathways results in enhanced transcriptional activity of the ER (17). Secondly, growth factors and growth factor receptor expression stimulation by steroids would additionally sensitize the cells to growth factors. Finally, steroid-dependent activation of MAPK and other intracellular kinases through nongenomic mechanisms would facilitate growth factor signaling pathways during periods of low level of growth factors (2).

Unlike its action in breast tissue, tamoxifen does not display antiproliferative action in endometrium and instead displays properties of an estrogen to promote proliferation. Because of this, patients undergoing tamoxifen therapy in either the chemoprevention or adjuvant setting for breast cancer are at...
risk for developing endometrial cancer (Refs. 18, 19 and references therein).

Endometrial cancer is one of the most common malignancies in postmenopausal women (20). Most carcinomas are grade I (well differentiated). The poorly differentiated grade 3 carcinomas exhibit both architectural and nuclear changes described with high grades (21). The progression of normal endometrium to endometrial cancer depends on many factors, the most important of which is estrogen. Endometrial hyperplasia could develop due to abnormal estrogen response, which is a precursor of estrogen-dependent endometrial carcinomas occurring mostly at a younger age and is most commonly endometrioid in its histological picture. Alternately, if the endometrium is estrogen independent (e.g., atrophic endometrium), serous or clear cell carcinomas may develop with very poor outcome (22).

No studies have examined MAPK and Src kinase expression and activity in malignant versus benign endometrium. These studies are of interest given that tamoxifen therapy for breast cancer is associated with development of endometrial carcinoma, and these kinases promote the agonist action of tamoxifen.

MATERIALS AND METHODS

Polyclonal antirabbit antibodies to both the total and to the phosphorylated (active) kinases for human p42/44 (ERK1/2), human p38, human SAPK/JNK MAPKs, and active Src kinase were purchased from Cell Signaling Technology, (Beverly, MA). Monoclonal antitumor antibody recognizing total Src was purchased from Santa Cruz, CA). Thirty-three freshly frozen, human endometrial adenocarcinoma (27 cases), papillary serous carcinoma (3 cases) were purchased from Vector Laboratories (Burlingame, CA). Anti-goat IgG (H+L), and horseradish peroxidase streptavidin were purchased from Vector Laboratories (Burlingame, CA). Antibody to beta-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Thirty-three freshly frozen, human endometrial adenocarcinomas and 38 benign endomyometrial tissue (26 proliferative and 12 secretory) were obtained from Cooperative Human Tissue Network. The histological types were endometrioid adenocarcinoma (27 cases), papillary serous carcinoma (3 cases), and clear cell carcinoma (3 case). Nine cases were grade I, 10 cases were grade II, and 14 cases were grade III. The tissues were immediately snap frozen in liquid nitrogen after resection to minimize protein degradation and dephosphorylation of the kinases. Each endometrial tissue (0.2–1.5 g) was cut into two to four equal sized pieces on dry ice with a razor blade and stored separately at –80°C. One section was used for homogenization for total protein extraction. An adjacent section was used for immunohistochemistry for active and total kinases, and for H&E staining to pathologically characterize the tissue. For protein extraction, ~0.1 g of tissue was homogenized in 600 µl of lysis buffer containing protease and phosphatase inhibitors [10 mM Tris (pH 8.0), 0.4 mM sodium chloride, 50 mM potassium phosphate, 50 mM sodium fluoride, 10 mM sodium molybdate, 2 mM EDTA, 2 mM EGTA, 1 mM sodium vanadate, 0.1% Triton-X 100, 10 mM β-mercaptoethanol, protease inhibitor (Sigma), and 0.1 mM phenylmethylsulfonyl fluoride]. Tissue was homogenized for protein extraction with a polystrom homogenizer (Kinematica AG, PT 1300 D; probe PT-DA 1305/2EC) at 30,000 rpm for 10–20 s on ice. The lysate was centrifuged at 21,000 × g for 10 min at 4°C. Protein concentration of the supernatant was measured by the Bradford Assay before addition of Laemli sample buffer.

Western Blot Analysis. One hundred µg of each protein extract was separated by electrophoresis on a 10% SDS-PAGE gel. The gel was transferred to nitrocellulose membrane and blocked in 5% nonfat milk in Tris-buffered saline/Tween 20 [TBST; 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] for 1 h at room temperature, followed by washes with 1× TBST. The membranes were incubated with each primary antibody overnight in 5% dried milk/TBST at 4°C. Total Src kinase antibody was used at 1 µg/ml, and a 1:1000 dilution of manufacturer-supplied concentration was used for all of the other primary antibodies. After primary antibody incubation, the membranes were washed with TBST and incubated with secondary biotinylated antirabbit IgG (1.5 µg/ml) for 1 h at room temp. After TBST washes, the membranes were incubated with horseradish peroxidase streptavidin (1 µg/ml) for 1 h at room temperature and washed with TBST. The signal was developed with addition of enhanced chemiluminescence solution [1:1 mixture of solution A+B [A: 9.9 ml of 0.1 mM Tris (pH 8.5) + 100 µl luminol +44 µl P-coumaric; B: 10 ml of 0.1 mM Tris (pH 8.5) + 6 µl hydrogen peroxide]}. The membranes were exposed to Kodak XOMAT film for 2 min, and the signal was quantitated using a Kodak imager as described below. Samples that showed no signal were reloaded (twice) to assure there were no problems with the Western procedure. To assess for protein degradation and to normalize protein load among samples, the membranes were incubated with antibody against β-actin after stripping the blots (see below). No tissue extracts exhibited degradation of β-actin.

Stripping of Membranes. After performing a Western blot using antibody to the kinases, the membranes were incubated in stripping buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol] for 30 min at 55°C with rocking to remove bound antibodies and prepare the membrane to be reprobed with antibody against β-actin (1 µg/ml) as described above.

Quantitation of the Enhanced Chemiluminescence Signal. The enhanced chemiluminescence signal was quantitated using a Kodak Digital Science one-dimensional image analysis software station 440 system. To compare the level of activated kinase among samples, the signal measured for the activated kinase was divided by the value for the total kinase to normalize for total kinase level and then this value was divided by the value for β-actin to normalize for protein load. To compare the level of total kinase among samples, the value for the total kinase was divided by the value for β-actin.

H&E Staining and Immunohistochemistry. One piece of tissue from each case was processed for paraffin blocks after fixation in 10% formalin. Four-μm thick sections were prepared on poly-l-lysine glass slides for H&E staining. Other sections from cases showing higher levels of kinases were prepared for immunohistochemistry for active and total kinases to study the spacial pattern of kinase expression in epithelial versus stromal cells. Tissue sections were deparaffinized by heating at 65°C for 15 min followed by successive 5-min incubations with xylene, 100% ethanol, 95% ethanol, dH2O, and PBS. For antigen retrieval and endogenous peroxidase quenching, the slides were
heated for 10 min in 10 mM sodium citrate buffer (pH 6.0) at 95°, cooled for 20 min (room temperature), and incubated in successive washes of dH₂O, 1% hydrogen peroxide (H₂O₂) for 10 min, dH₂O, and PBS. Tissue sections were blocked with 10% goat serum in PBS including 4 drops/ml of avidin D (Vector Laboratories) for 45 min. The slides were incubated with primary antibody in 10% goat serum in PBS + 4 drops of biotin solution/ml (Vector Laboratories) overnight at 4° (total Src kinase antibody was used at 10 μg/ml). A 1:100 dilution of manufacturer-supplied concentration for all of the other kinases was used except active and total p38 antibody used at a 1:50 dilution. The slides were incubated with 3 × 5 min washes with PBS and then incubated with secondary antibody in 10% goat serum in PBS for 45 min (15 μg/ml) followed by streptavidin peroxidase in 10% goat serum for 45 min (10 μg/ml). Color was developed by incubating slides with 3, 3′-diaminobenzidine

Fig. 1 H&E stain of representative endometrial tissue sections. Proliferative phase endometrium, showing uniform tubular glands lined by one layer of columnar cells surrounded by cellular stroma (A). Secretory phase endometrium showing tortuous glands lined by one layer of cubical epithelium containing secretory vacuoles surrounded by edematous stroma (B). Endometrioid adenocarcinoma, grade I showing closely packed, pleomorphic glands lined by columnar epithelium (C). Endometrioid carcinoma grade III showing closely packed solid sheets of malignant, markedly pleomorphic cells with hyper-chromatic nuclei and abnormal mitoses surrounded by desmoplastic stroma (D). Bar = 50 micrometers. Magnifications are ×200.
Fig. 2  Expression of extracellular signal-regulated kinase (ERK) 1/2 kinase in benign and malignant endometrium. Endometrial tissue representing 38 benign proliferative and secretory \( (n = 38; \text{mean age, 42}) \) and 33 carcinoma \( (n = 33; \text{Grade 1 } (n = 9), \text{Grade 2 } (n = 10), \text{and Grade 3 } (n = 14)) \) were homogenized in lysis buffer, and 100 \( \mu \text{g} \) of tissue extract was separated by 10\% SDS-PAGE and prepared for Western blotting with antibodies against total and phosphorylated forms of ERK1/2 kinase and antibody to \( \beta \)-actin. Western blot signals were digitally quantitated using Kodak Image Station 440CF and 1D Image Analysis software. Quantitated signals are plotted in A. Representative Western blots are shown in B \((42 \text{ and } 44 \text{ kDa})\); bars, \( \pm \text{SE} \). Statistical comparison between total benign \( (n = 38) \) and total carcinoma \( (n = 33) \) was performed using the Mann-Whitney \( U \) test. Statistical comparison between the different grades of endometrial adenocarcinomas was calculated by Kruskal-Wallis \( H \) test. **, \( P \leq 0.05 \); *, \( P \leq 0.001 \).

Fig. 3  Expression of Src tyrosine kinase in normal and malignant endometrium. Endometrial tissue representing 38 benign proliferative and secretory \( (n = 38; \text{mean age, 42}) \) and 33 carcinoma \( (n = 33; \text{Grade 1 } (n = 9), \text{Grade 2 } (n = 10), \text{and Grade 3 } (n = 14)) \) were homogenized in lysis buffer, and 100 \( \mu \text{g} \) of tissue extract was separated by 10\% SDS-PAGE and prepared for Western blotting with antibodies against total and phosphorylated forms of Src kinase or antibody to \( \beta \)-actin. Western blot signals were digitally quantitated using Kodak Image Station 440CF and 1D Image Analysis software. Quantitated signals are plotted in A. Representative Western blots are shown in B \((60 \text{ kDa})\); bars, \( \pm \text{SE} \). Statistical comparison between total benign \( (n = 38) \) and total carcinoma \( (n = 33) \) was performed using the Mann-Whitney \( U \) test. Statistical comparison between the different grades of endometrial adenocarcinomas was calculated by Kruskal-Wallis \( H \) test. *, \( P \leq 0.001 \).
Fig. 4  Expression of p38 kinase in normal and malignant endometrium. Endometrial tissue representing 38 benign proliferative and secretory \((n = 38; \text{mean age, 42})\) and 33 carcinoma \((n = 33; \text{Grade 1 } (n = 9), \text{Grade 2 } (n = 10), \text{and Grade 3 } (n = 14))\) were homogenized in lysis buffer, and 100 \(\mu\)g of tissue extract was separated by 10% SDS-PAGE and prepared for Western blotting with antibodies against total and phosphorylated forms of p38 kinase or antibody to \(\beta\)-actin. Western blot signals were digitally quantitated using Kodak Image Station 440CF and 1D Image Analysis software. Quantitated signals are plotted in A. Representative Western blots are shown in B (43 kDa); bars, ±SE. Statistical comparison between total benign \((n = 38)\) and total carcinoma \((n = 33)\) was performed using the Mann-Whitney \(U\) test. Statistical comparison between the different grades of endometrial adenocarcinomas was calculated by Kruskal-Wallis \(H\) test. *, \(P \leq 0.001\).

Fig. 5  Expression of stress-activated protein kinase/c-Jun NH\(_2\)-terminal kinase (SAPK/JNK) kinase in normal and malignant endometrium. Endometrial tissue representing 38 benign proliferative and secretory \((n = 38; \text{mean age, 42})\) and 33 carcinoma \((n = 33; \text{Grade 1 } (n = 9), \text{Grade 2 } (n = 10), \text{and Grade 3 } (n = 14))\) were homogenized in lysis buffer, and 100 \(\mu\)g of tissue extract was separated by 10% SDS-PAGE and prepared for Western blotting with antibodies against total and phosphorylated forms of SAPK/JNK kinase or antibody to \(\beta\)-actin. Western blot signals were digitally quantitated using Kodak Image Station 440CF and 1D Image Analysis software. Quantitated signals are plotted in A. Representative Western blots are shown in B (46 and 54 kDa); bars, ±SE. Statistical comparison between total benign \((n = 38)\) and total carcinoma \((n = 33)\) was performed using the Mann-Whitney \(U\) test. Statistical comparison between the different grades of endometrial adenocarcinomas was calculated by Kruskal-Wallis \(H\) test. *, \(P \leq 0.001\).
Table 1  Expression of MAPKs\textsuperscript{a} and Src kinase in benign and malignant endometrium

Statistical difference in the distribution of active and total MAPKs and Src kinase in benign endometrium and endometrial adenocarcinomas was calculated by the Mann-Whitney U test. Values shown are the mean absolute numbers obtained from Kodak image analysis of the Western blot signals. To determine the level of active kinase, the activated kinase value was divided by total kinase value and the value for β-actin. To determine the level of total kinase, the total kinase value was divided by the β-actin value. All values shown are ± SE of the mean.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Benign</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>PERK/TERK/actin</td>
<td>151 ± 40</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>TERK/actin</td>
<td>0.7 ± 0.1</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>PSrc/TSrc/actin</td>
<td>28 ± 4</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>TSrc/actin</td>
<td>0.4 ± 0.1</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Pp38/Tp38/actin</td>
<td>132 ± 16</td>
<td>27 ± 7</td>
</tr>
<tr>
<td>Tp38/actin</td>
<td>0.4 ± 0.1</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>PJNK/TJNK/actin</td>
<td>44 ± 10</td>
<td>52 ± 13</td>
</tr>
<tr>
<td>TJNK/actin</td>
<td>0.2</td>
<td>2.0 ± 0.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH\textsubscript{2}-terminal kinase.

\textsuperscript{b} Statistically significant.

Table 2  Expression of MAPKs\textsuperscript{a} and Src kinase in proliferative and secretory phase endometrium (benign group)

Statistical difference in the distribution of MAPKs and Src kinase in proliferative and secretory phase endometrium was calculated by the Mann-Whitney U test. Values shown are the mean absolute numbers obtained from Kodak image analysis of the Western blot signals. To determine the level of active kinase, the activated kinase value was divided by total kinase value and the value for β-actin. To determine the level of total kinase, the total kinase value was divided by the β-actin value. All values shown are ± SE of the mean.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Proliferative</th>
<th>Secretory</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PERK/TERK/actin</td>
<td>161 ± 55</td>
<td>127 ± 43</td>
<td>0.5</td>
</tr>
<tr>
<td>TERK/actin</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>PSrc/TSrc/actin</td>
<td>25 ± 5</td>
<td>34 ± 9</td>
<td>0.4</td>
</tr>
<tr>
<td>TSrc/actin</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Pp38/Tp38/actin</td>
<td>121 ± 20</td>
<td>154 ± 24</td>
<td>0.3</td>
</tr>
<tr>
<td>Tp38/actin</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>PJNK/TJNK/actin</td>
<td>34 ± 10</td>
<td>64 ± 23</td>
<td>0.2</td>
</tr>
<tr>
<td>TJNK/actin</td>
<td>0.2 ± 0.03</td>
<td>0.2 ± 0.04</td>
<td>0.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH\textsubscript{2}-terminal kinase.

RESULTS

All of the tissue specimens stained with H&E were histologically evaluated by a pathologist (M. D.). In normal proliferative endometrium (26 cases; Fig. 1A), the glands were uniform, tubular, lined by one layer of columnar cells, showing frequent normal mitoses, and surrounded by dense cellular stroma. In normal secretory endometrium (12 cases; Fig. 1B), the histological features were tortuous glands lined by one layer of cubical epithelial cells containing secretory vacuoles, surrounded by edematous, congested stroma with few poly morphonuclear leukocyte infiltration, and coiling of the arterioles.

The histological pictures of endometrial carcinomas (33 cases) were diagnosed according to the international histological clas-

Table 3  Expression of MAPKs\textsuperscript{a} and Src kinase in different grades of endometrial carcinomas

Statistical differences in the distribution of MAPKs and Src kinase in different grades of endometrial adenocarcinomas was calculated by the Kruskal-Wallis H test. Values shown are the mean absolute numbers obtained from Kodak image analysis of the Western blot signals. To determine the level of active kinase, the activated kinase value was divided by total kinase value and the value for β-actin. To determine the level of total kinase, the total kinase value was divided by the β-actin value. All values shown are ± SE of the mean.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Grade I (n = 9)</th>
<th>Grade II (n = 10)</th>
<th>Grade III (n = 14)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PERK/TERK/actin</td>
<td>51 ± 17</td>
<td>40 ± 9</td>
<td>42 ± 12</td>
<td>0.07</td>
</tr>
<tr>
<td>TERK/actin</td>
<td>9 ± 2</td>
<td>9 ± 2</td>
<td>9 ± 4</td>
<td>0.6</td>
</tr>
<tr>
<td>PSrc/TSrc/actin</td>
<td>3 ± 2</td>
<td>4 ± 2</td>
<td>4 ± 1</td>
<td>0.8</td>
</tr>
<tr>
<td>TSrc/actin</td>
<td>5 ± 2</td>
<td>9 ± 5</td>
<td>6 ± 2</td>
<td>0.5</td>
</tr>
<tr>
<td>Pp38/Tp38/actin</td>
<td>35 ± 20</td>
<td>23 ± 10</td>
<td>22 ± 8</td>
<td>0.3</td>
</tr>
<tr>
<td>Tp38/actin</td>
<td>1 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>1 ± 0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>PJNK/TJNK/actin</td>
<td>55 ± 17</td>
<td>54 ± 32</td>
<td>47 ± 19</td>
<td>0.4</td>
</tr>
<tr>
<td>TJNK/actin</td>
<td>1 ± 0.4</td>
<td>3 ± 1</td>
<td>1 ± 0.5</td>
<td>0.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a} MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH\textsubscript{2}-terminal kinase.

Table 4  Expression of MAPKs\textsuperscript{a} and Src kinase in serous and clear cell carcinoma and grade 3 endometrioid adenocarcinoma

Statistical difference in the distribution of MAPKs and Src kinase in serous and clear cell carcinoma and grade 3 endometrioid adenocarcinoma was calculated by the Mann-Whitney U test. Values shown are the mean absolute numbers obtained from Kodak image analysis of the Western blot signals. To determine the level of active kinase, the activated kinase value was divided by total kinase value and the value for β-actin. To determine the level of total kinase, the total kinase value was divided by the β-actin value. All values shown are ± SE of the mean.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Serous and clear cell carcinoma (n = 6)</th>
<th>Grade 3 endometrioid cell carcinoma (n = 9)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PERK/TERK/actin</td>
<td>36.0 ± 14.7</td>
<td>34.1 ± 11.4</td>
<td>0.6</td>
</tr>
<tr>
<td>TERK/actin</td>
<td>6.33 ± 2.6</td>
<td>10.7 ± 3.6</td>
<td>0.9</td>
</tr>
<tr>
<td>PSrc/TSrc/actin</td>
<td>3.17 ± 1.3</td>
<td>4.50 ± 1.5</td>
<td>0.4</td>
</tr>
<tr>
<td>TSrc/actin</td>
<td>2.17 ± 0.9</td>
<td>7.23 ± 2.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Pp38/Tp38/actin</td>
<td>25.2 ± 10.3</td>
<td>20.1 ± 6.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Tp38/actin</td>
<td>0.34 ± 0.1</td>
<td>1.09 ± 0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>PJNK/TJNK/actin</td>
<td>23.2 ± 9.5</td>
<td>45.7 ± 15.2</td>
<td>0.3</td>
</tr>
<tr>
<td>TJNK/actin</td>
<td>2.96 ± 1.2</td>
<td>1.25 ± 0.4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH\textsubscript{2}-terminal kinase.
Fig. 6  Immunohistochemistry for active and total Src and mitogen-activated protein kinases (MAPKs) from representative benign and malignant endometrial tissue sections that showed high kinase expression by Western blot. Four μm-thick paraffin sections were incubated with primary antibodies against active Src (A) in a grade I endometrioid adenocarcinoma case showing positive brown staining mainly in the cytoplasm of epithelial
sification (23), including endometrioid adenocarcinoma (27 cases; Fig. 1, C and D), papillary serous carcinoma (3 cases), and clear cell carcinoma (3 cases). The cases were graded according to standard pathological grading system (21), including grade I (9 cases; Fig. 1C), grade II (10 cases), and grade III (14 cases; Fig. 1D). The mean age of endometrial adenocarcinomas was 64 ± 3 (range, 30–85 years); grade I, grade II, and grade III were a mean age of 65 ± 4, 57 ± 5, and 69 ± 4, respectively, whereas for normal endometrium specimens the mean age was 42 ± 2, proliferative 42 ± 1, and secretory was 40 ± 1.

We found elevated active ERK1/2 (151 ± 40. versus 46 ± 7; Fig. 2), Src (28 ± 4 versus 4 ± 1; Fig. 3), and p38 (132 ± 16 versus 27 ± 7; Fig. 4) in benign versus malignant endometrium, respectively, when the absolute Western blot signal of activated kinase was normalized to total kinase and β-actin using Kodak image analysis as described in “Materials and Methods.” A modest increase in active SAPK/JNK was detected in carcinoma (52 ± 13 versus 44 ± 10; Fig. 5). For each kinase, expression of total kinase (normalized to β-actin) was higher in carcinoma relative to benign endometrium, respectively (ERK1/2, 9 ± 2 versus 0.7 ± 0.1, Fig. 2; Src, 7 ± 2 versus 0.4 ± 0.1, Fig. 3; p38, 1 ± 0.2 versus 0.4 ± 0.1, Fig. 4; and SAPK/JNK, 2 ± 0.4 versus 0.2 ± 0.02, Fig. 5; Table 1). There was no significant difference between the distribution of total and activated kinases in the proliferative and secretory phases of endometrium in benign tissues (Table 2), as well as between the different grades of the endometrial carcinomas (Table 3).

Serous and clear cell carcinomas are distinct from endometrioid type tumors at both the pathological and molecular level. We compared the total and activated kinase expression between serous (3 cases) and clear cell (3 cases) carcinomas collectively in comparison with grade 3 endometrioid adenocarcinoma (9 cases) using Western blotting quantitation data. In the serous/clear cell group, 5 cases were grade 3, and 1 clear cell carcinoma case was grade 2. There was no significant difference between the distribution of total and activated kinases in the serous/clear cell carcinoma cases and the grade 3 endometrioid adenocarcinomas (Table 4). Because of the low sample number, additional studies should be performed to confirm if differences exist.

Tissues from representative benign (n = 5) and malignant (n = 8) cases that exhibited high kinase expression by Western blotting (see Figs. 2–5) were selected for immunohistochemistry of paraffin sections with the same antibodies used in the Western blots per the manufacturer’s procedures. Specimens stained with antibody to active Src (Fig. 6A) and total Src (data not shown) showed positive staining mainly in the cytoplasm of epithelial and stroma cells. MAPKs [active ERK1/2 (Fig. 6B), total ERK1/2 (Fig. 6C), active p38 (data not shown), total p38 (Fig. 6D), active SAPK/JNK (data not shown), and total SAPK/JNK (Fig. 6E)], also showed positive staining mainly in the epithelial cells and to lesser extent in stroma cells (data summarized in Table 5). Negative control immunohistochemistry showed no positive staining in either epithelial or stromal cells (Fig. 6F).

**DISCUSSION**

Previous studies have detected an increase in activated MAPKs and Src kinase during progression of cancers. In breast, increased levels of active phosphorylated MAPKs ERK1/2, p38, SAPK/JNK (24), and tyrosine kinase Src (16) were detected in breast carcinomas versus normal benign breast tissue. In the present work we measured both total and active phosphorylated kinase levels for ERK1/2, p38, and tyrosine kinase Src (16) were detected in breast carcinomas versus normal benign breast tissue. In the present work we measured both total and active phosphorylated kinase levels for ERK1/2, p38, and tyrosine kinase Src (16) were detected in breast carcinomas versus normal benign breast tissue.

We compared the total and activated kinase expression between serous (3 cases) and clear cell (3 cases) carcinomas collectively in comparison with grade 3 endometrioid adenocarcinoma (9 cases) using Western blotting quantitation data. In the serous/clear cell group, 5 cases were grade 3, and 1 clear cell carcinoma case was grade 2. There was no significant difference between the distribution of total and activated kinases in the serous/clear cell carcinoma cases and the grade 3 endometrioid adenocarcinomas (Table 4). Because of the low sample number, additional studies should be performed to confirm if differences exist.

Tissues from representative benign (n = 5) and malignant (n = 8) cases that exhibited high kinase expression by Western blotting (see Figs. 2–5) were selected for immunohistochemistry of paraffin sections with the same antibodies used in the Western blots per the manufacturer’s procedures. Specimens stained with antibody to active Src (Fig. 6A) and total Src (data not shown) showed positive staining mainly in the cytoplasm of epithelial and stroma cells. MAPKs [active ERK1/2 (Fig. 6B), total ERK1/2 (Fig. 6C), active p38 (data not shown), total p38 (Fig. 6D), active SAPK/JNK (data not shown), and total SAPK/JNK (Fig. 6E)], also showed positive staining mainly in the epithelial cells and to lesser extent in stroma cells (data summarized in Table 5). Negative control immunohistochemistry showed no positive staining in either epithelial or stromal cells (Fig. 6F).

**DISCUSSION**

Previous studies have detected an increase in activated MAPKs and Src kinase during progression of cancers. In breast, increased levels of active phosphorylated MAPKs ERK1/2, p38, and tyrosine kinase Src (16) were detected in breast carcinomas versus normal benign breast tissue. In the present work we measured both total and active phosphorylated kinase levels for ERK1/2, p38, SAPK/JNK (24), and tyrosine kinase Src (16) were detected in breast carcinomas versus normal benign breast tissue. In the present work we measured both total and active phosphorylated kinase levels for ERK1/2, p38, SAPK/JNK (24), and tyrosine kinase Src (16) were detected in breast carcinomas versus normal benign breast tissue.
endometrial specimens in different phases of menstrual cycle by quantitation of signals from Western blots using antibodies against the active and total kinases. In contrast to results reported for breast carcinoma, we found that active ERK1/2 was elevated 3-fold in benign endometrium versus endometrial carcinomas. Active Src was elevated 7-fold in benign versus carcinomas. Active p38 was elevated 5-fold in normal versus carcinomas. Active SAPK/JNK was not significantly altered in endometrial carcinomas versus benign tissue.

Immunohistochemistry experiments examined the spacial distribution of kinases between the endometrial stroma and epithelium. We detected positive staining for all of the kinases mainly in epithelial cells and to a lesser extent in stromal cells. For ERK1/2 a similar spacial expression in prostatic epithelium and stroma was detected by Uzgare et al. (25), although this study detected active p38 only in glandular epithelial cells with no positive stromal staining. Our data for active and total Src kinase expression mainly in the cytoplasm of epithelial and stromal cells is similar to what was found by Reissig et al. (16), who also demonstrated positive staining for active Src in both glandular epithelium and stromal compartments of breast carcinoma tissues. Yamamoto et al. (26) found that in proliferative endometrium, active Src expression was detected mainly in glandular epithelium and that stromal Src exhibited perinuclear localization. In secretory phase endometrium, active Src was detected in both the stroma and glandular epithelium (26). Our data showed that active and total SAPK/JNK exhibited positive staining mainly in epithelial cells and to a lesser extent in stromal cells. Gee et al. (27) and Wang et al. (24) demonstrated similar positive nuclear staining for active SAPK/JNK in breast carcinoma.

With regard to tamoxifen therapy, our findings are of interest given that tamoxifen therapy for breast carcinoma may lead to development of abnormal endometrial proliferation that may progress to endometrial carcinoma. In agreement with results reported for breast cancer, the levels of total kinase were found to be elevated in endometrial cancers versus normal endometrium. As reported by Si-tometrium. These findings may be explained as reported for breast cancer, the levels of total kinase were elevated with endometrial malignancy, there was no elevation in the level of active kinase. Although it remains to be determined whether the net effect of active kinase in normal versus malignant endometrium is altered, the malignant endometrium is clearly different from breast cancer tissues in which both the level and the activity of these kinases rise with malignancy.

Elevation of active kinases in benign endometrium could contribute to the endometrial resistance to tamoxifen antagonist action. Estrogen is a well-known proliferative stimulus for glandular epithelial cells in both the endometrium and breast tissue under normal and pathological conditions. The major mechanism for the antiproliferative action of tamoxifen is as an ER antagonist. Tamoxifen prevents breast cancer proliferation by competing with estrogens for binding to the ER. However, tamoxifen exhibits an estrogen agonist action in the endometrium and stimulates endometrial proliferation likely through the stromal compartment (29). This observation could explain the increased incidence of abnormal endometrial proliferations (polyps, hyperplasia, and carcinoma) in postmenopausal tamoxifen users (30). Cell culture studies have shown that tamoxifen agonist action is promoted by active Src and MAPKs in part through phosphorylation of ERα and its associated coagonulators (31, 32). In one example using the Ishikawa endometrial cancer cells where tamoxifen functions as an agonist, it was found that active p38 in turn activates ERα through phosphorylation of the kinase JNK-311. This phosphorylation promoted both nuclear retention of the receptor and interaction with coactivators (33). With respect to active kinase levels, tamoxifen resistance in normal endometrium may be similar to tamoxifen resistance in advanced breast cancer (31, 34). Both tissues display high Src and MAPK activity. Additional studies will be needed to determine the significance of kinase activity between benign and malignant endometrium with regard to tumor growth and ER action.

Normal cycling (premenopausal) endometrium was used for comparison to malignant endometrium in this study. In the cycling tissue, there was no significant difference in the expression of kinases studied between proliferative and secretory endometrium. However, it would be important in future studies to examine kinase expression in inactive (postmenopausal) endometrium and compare this to malignant endometrium because endometrial cancers mainly arise in postmenopausal patients. To our knowledge, kinase activity in noncycling endometrium has not been examined. In addition, it will be important to determine whether there is a difference in the expression of kinases in pre-versus postmenopausal normal endometrium.

REFERENCES
Src Kinase and Mitogen-Activated Protein Kinases in the Progression from Normal to Malignant Endometrium

Mohamed Mokhtar Desouki and Brian G. Rowan


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/2/546

Cited articles
This article cites 30 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/2/546.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/10/2/546.full.html#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.