Original Article
SHP$_2$ is an independent negative prognostic factor for endometrial cancer

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Abstract: Aim: To investigate the prognostic significance of Src homology 2 domain-containing phosphatase (SHP$_2$) in endometrial cancer (EC). Methods and Results: The mRNA expression levels of SHP$_2$ of 35 fresh EC samples and 15 normal endometrium samples were detected by Real-Time RT-PCR. A microarray tissue including 110 EC paraffin-embedded samples and 20 normal endometrium paraffin-embedded samples was detected by the immunohistochemistry. The mRNA expression level of SHP$_2$ in EC tissues was higher than that of normal endometrium (Mann-Whitney $U=38$, $P<0.05$). Higher positive rates of SHP$_2$ expression were observed in patients with lower grade ($\chi^2=7.102$, $P=0.008$), lymph node metastases ($\chi^2=7.102$, $P=0.008$) and myometrial invasion ($\chi^2=5.669$, $P=0.017$). The positive expression of SHP$_2$ correlated significantly with that of EGFR ($r=0.853$, $P<0.001$). The Cox proportional hazards regression indicated SHP$_2$ was an independent prognostic factor (HR=7.906, 95% CI=1.379-45.308, $P<0.05$). ROC curve indicated SHP$_2$ combined with other clinicopathological risk factors was a significantly stronger prognostic model (AUC=0.883, 95% CI=0.771-0.996). Conclusions: SHP$_2$ can be regarded as an independent negative prognostic factor for endometrial cancer.

Keywords: SHP$_2$, endometrial cancer, prognosis

Introduction

Endometrial cancer (EC) is the most common gynecologic cancer among women in the United States, with roughly 54,000 new patient cases expected in 2015 [1]. It is closely related to the increasing population with overweight or obesity [2]. Due to earlier detection, tumor surgery and adjuvant treatment, the overall survival of these patients has markedly been improved, but the prognosis remains poor in cases of advanced or recurrent EC. Classification of EC by morphologic features is inconsistent, and yields limited predictive and prognostic information. Therefore, extensive studies have focused on the identification of molecular predictors and potential targets for this disease.

Src homology 2 (SHP$_2$) domain-containing phosphatase 2 (SHP$_2$), encoded by PTPN11, is a first known proto-oncogene in the PTP (protein-tyrosine phosphatase) super-family. It is hyperactivated either by downstream of oncoproteins or by mutations in many tumors, such as breast cancer [3], lung cancer [4], gastric cancer [5], cervical cancer [6] and prostate cancer [7]. Signaling pathways involving SHP$_2$ have also been discovered. Many studies have shown that SHP$_2$ is essential for the growth factor-mediated Ras-Raf-ERK pathway [8] and is a positive regulator of PI3K-AKT pathway [9]. In addition, SHP$_2$ has been indicated in JAK/STAT, JNK and NF-$\kappa$B signaling, which also have strong associations with various tumors [10]. Meanwhile, some research observed SHP$_2$ may promote tumor metastases by regulating epithelial-mesenchymal transition (EMT) [11-13] and suppress oxidative stress and senescence in some tumors by enhances mitochondrial metabolism [14, 15]. Recent years, there has been renewed interest in developing SHP$_2$ inhibitors as potential anticancer agents [16, 17]. As an important downstream effector in EGFR family signaling, SHP$_2$ may represent an effective strategy for treatment of EGFR inhibitor resistant non-small cell lung cancer [18, 19] and HER2-positive breast cancer [20]. It is known that the EGFR/HER family has also been associated with the development and progression of EC [21]. However, the specific role of
SHP$_2$ expression in endometrial cancer

Figure 1. SHP$_2$ mRNA expression in 35 fresh EC tissue samples and 15 fresh normal endometrial tissues using real-time quantitative RT-PCR. SHP$_2$ mRNA expression of EC is higher than that of normal endometrium.

Stimulated by the above observation, we investigated the expression of SHP$_2$ in EC, analyzed the correlation between SHP$_2$ and clinicopathological parameters, and assessed its prognostic value for overall survival of EC patients. Furthermore, we explored the potential correlation between SHP$_2$ and EGFR protein staining.

Materials and methods

Samples and clinical database

All EC and normal endometrial tissue samples were obtained during January 2008 and March 2013 from Shanghai Jiao Tong University affiliated Sixth People's Hospital. Thirty-five fresh EC samples and fifteen fresh normal endometrial samples were obtained immediately after the surgical procedure. One hundred-ten FFPE (formalin fixed and paraffin-embedded) EC samples and twenty FFPE normal endometrial samples were constructed tissue microarrays by Zuoli Biotechnology Company (Shanghai, China), which were also employed in our previous publications [22, 23].

Patients were excluded if they underwent radiotherapy, chemotherapy, or other therapies prior to surgery. All patients had completed informed consented approval of the ethics committee of Shanghai Jiao Tong University affiliated Sixth People's Hospital for the use of samples. Clinical data, including tumor classification, lymph node metastasis and vascular invasion were collected from medical records. Among all patients, the mean age was 57 years. Ninety-seven patients were identified at FIGO stage I, 9 at stage II, and 4 at stage III. Sixty-seven samples were defined as well-differentiated (G1), 30 moderately-differentiated (G2) and 13 poorly-differentiated (G3). Patients were followed up by out-patient clinic medical records or telephone calls. Overall survival was defined as the time from the date of surgery to the date of the last follow-up examination or death.

Real-time quantitative RT-PCR analysis

Primer sequences used for RT-PCR were given as follows: β-actin: sense 5’-GCTTCTGTTCC-GATGATA-3’, anti-sense 5’-CCTGCCACACCTCATC-TCTG-3’; SHP$_2$: sense 5’-GAGAACGGGUUUGA-UUCUUTT-3’, anti-sense 5’-AAGAAUCAAACCGUUCUCCCT-3’). Real-time PCR was performed using LightCycler® FastStart DNA Master SYBR Green I (Analytik Jena, Germany) according to the manufacturer’s instructions. The PCR condition was designed as 40 cycles of denaturing (95°C for 15 seconds), annealing (60°C for 15 seconds) and extension (72°C for 30 seconds). β-actin was amplified as an internal control.

Immunohistochemistry

We deparaffinized the slides in xylene for 10 min per time for three times, rehydrated with a graded series of ethanol concentrations (in 100%, 95%, 85%, 75% ethanol for 10 min respectively) and performed antigen retrieval in 100°C water with 0.01 M citrate buffer for 30 minutes. The sections were incubated in 37°C with 0.3% hydrogen peroxide/phosphate-buffered saline for 30 minutes and blocked at room temperature for 1 hour. SHP$_2$ antibody (1:1000, CST, USA) and EGFR antibody (1:100, EPITOMICS, USA) were incubated overnight at 4°C. We visualized the slides using DAB substrate liquid (Thermo Scientific USA), washed them with deionized water before hematoxylin counterstaining. The percentage of positive staining (P) were scored as 0 (<25%), 1 (25%-50%), 2 (51%-75%), and 3 (76%-100%), and the levels of intensity of staining (I) were determined as 0, negative; 1, weak staining; 2, moderate staining; and 3, strong staining. The total scores (S) were designated as P × I for each section. According to the percentage of positive cells and staining intensity, the level of the protein expression was divided into three groups by the product of the two scores above: 0-1 (negative expression), 2-4 (low expression) and 5-9 (high expression).
Table 1. Relationship between SHP2 expression and clinicopathological features of endometrial cancer patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number (n)</th>
<th>SHP2 positive (n, %)</th>
<th>SHP2 negative (n, %)</th>
<th>χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical pathological stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>97</td>
<td>51 (52.6%)</td>
<td>46 (47.4%)</td>
<td>5.001</td>
<td>0.082</td>
</tr>
<tr>
<td>II, III, IV</td>
<td>13</td>
<td>11 (84.6%)</td>
<td>2 (15.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological grading</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>67</td>
<td>31 (46.3%)</td>
<td>36 (53.7%)</td>
<td>7.102</td>
<td>0.008</td>
</tr>
<tr>
<td>G2 and G3</td>
<td>43</td>
<td>31 (72.1%)</td>
<td>12 (27.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>103</td>
<td>57 (55.3%)</td>
<td>46 (44.7%)</td>
<td>0.690</td>
<td>0.406</td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>5 (71.4%)</td>
<td>2 (28.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphatic metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>100</td>
<td>53 (53%)</td>
<td>47 (47%)</td>
<td>5.060</td>
<td>0.024</td>
</tr>
<tr>
<td>Yes</td>
<td>10</td>
<td>9 (90%)</td>
<td>1 (10%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myometrial invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>88</td>
<td>44 (50.6%)</td>
<td>43 (49.4%)</td>
<td>5.669</td>
<td>0.017</td>
</tr>
<tr>
<td>Yes</td>
<td>22</td>
<td>18 (78.3%)</td>
<td>5 (21.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>101</td>
<td>55 (54.5%)</td>
<td>46 (45.5%)</td>
<td>1.828</td>
<td>0.176</td>
</tr>
<tr>
<td>Squamous, papillary, clear cell carcinoma</td>
<td>9</td>
<td>7 (77.7%)</td>
<td>2 (22.3%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Expressions of SHP2 protein in endometrial samples. Original magnification ×100. A: No SHP2 expression in normal endometrium, defined as negative staining. B: SHP2 expression in endometrial cancer grade 1, defined...
SHP<sub>2</sub> expression in endometrial cancer

as positive staining. C: SHP<sub>2</sub> expression in endometrial cancer grade 2, defined as strong positive staining. D: SHP<sub>2</sub> expression in endometrial cancer grade 3, defined as strong positive staining.

![Graphs showing survival rates for different stages and conditions.](image)

**Figure 3.** Univariate analyses of factors associated with OS by Kaplan-Meier method and log-rank test.

**Table 2.** Multivariate analyses of factors associated with OS by the Cox proportional hazard regression model with step-wise manner (forward: condition, entry α=0.05, stay α=0.1)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OS hazard ratio</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>15.307</td>
</tr>
<tr>
<td>Myometrial invasion</td>
<td>10.716</td>
</tr>
<tr>
<td>SHP&lt;sub&gt;2&lt;/sub&gt; expression</td>
<td>7.906</td>
</tr>
<tr>
<td>Lymphatic metastasis</td>
<td>27.226</td>
</tr>
</tbody>
</table>

*P<0.05.

**Results**

Expression of SHP<sub>2</sub> is up-regulated in EC

SHP<sub>2</sub> mRNA expression of EC is higher than that of normal endometrium (**Figure 1**). SHP<sub>2</sub>-positive expression was observed in only 1 case (5%) in 20 normal endometrial samples while 62 cases (56.4%) were observed in 110 EC samples. These results suggested SHP<sub>2</sub> may be involved in cancer progression.

**Statistical analysis**

We conducted statistical analyses with SPSS 19.0 software (Chicago, IL, USA), using χ² test for enumeration data, Mann-Whitney U test for ranked data, analyzed the correlation between overall survival (OS) and variables using the Kaplan-Meier method and compared survival curves using the log-rank test for survival analyses. ROC curves were used to compare the prognostic accuracy of SHP<sub>2</sub> with other risk factors. Cox proportional hazard model was fit to identify factors significantly related to overall survival. All analyses were two-sided and significance was set at a P value of 0.05.
SHP₂ expression in endometrial cancer

As shown in Table 1 and Figure 2, SHP₂ expression level is closely correlated with grade, lymph node metastases and myometrial invasion. A univariate analysis demonstrated that OS rate of SHP₂-positive patients was obviously lower than that of SHP₂-negative patients (Figure 3). SHP₂ expression was an independent prognostic factor for OS of EC patients. Moreover, in stage I, G1, no vascular invasion, no myometrial invasion and no lymphatic metastasis group, OS of SHP₂-negative group was distinctly better than that of SHP₂-positive group.
SHP₂ expression in endometrial cancer

The sensitivity and specificity of SHP₂ for endometrial cancer prognosis

To further confirm the prognostic accuracy of SHP₂, an ROC curve was performed. Statistical analyses displayed that the area under the curve (AUC) was 0.690 for SHP₂, while combined with clinicopathological factors AUC increased to 0.883, which was higher than AUC for clinicopathological factor (Figure 5 and Table 3). So SHP₂ combined with clinicopathological factors had more sensitivity and specificity and could become a stronger prognostic factor for EC.

The protein level of SHP₂ is closely correlated with EGFR expression

To further explore the potential mechanism of SHP₂, we compared the relationship between SHP₂ and EGFR protein expression in EC samples. EGFR staining was localized predominantly to the membrane and cytoplasm. EGFR positive expression was observed in 66 cases (60%) and its expression was closely related with SHP₂ expression (r=0.853, P<0.001) (Table 4 and Figure 6).

Discussion

Recent years, target-based drug discovery has become the dominant strategy for cancer therapies. In this process, target identification and validation play an important role in the success of drug discovery project. Although there is accumulating evidence that SHP₂ plays a positive role in oncogenic signaling and tumorigenesis, and is considered to be a potential target for tumors [16], its role in EC has not been explored.

In our study, we found the expression of SHP₂ increased in EC tissue compared to normal endometrium, either in mRNA level or in protein level, which indicated that SHP₂ may play important role in EC. SHP₂ is essential to the balance between ERK and PI3K/AKT and STAT
SHP2 expression in endometrial cancer

activity [24], which are all well recognized onco-
genic pathways. Knockdown of SHP2 partially inhibited proliferation of cancer cells in vitro, and decreased tumor growth and lung metas-
tases in a mouse model [25, 26]. Consistent with these studies, we found a positive associa-
tion between SHP2 expression, tumor grade, and lymph node metastasis in EC, which con-
ferred an important role for SHP2 in tumor ini-
tiation, progression and metastasis [27]. How-
ever, in contrast with Muenst et al. [28], no sig-
nificant association was found with tumor stage in our study, which could be accounted for the limited cases in advanced tumor stage. Of note, SHP2 expression proved to be an inde-
pendent negative prognostic factor for overall survival in EC. Although negative prognostic value of SHP2 has already been found in breast cancer [3], colorectal cancer [28] and live can-
cer [29], this is the first report to assess the impact of SHP2 expression on prognosis in EC. In subgroup analyses, SHP2 expression was associated with worse overall survival in patients with stage I, grade1, no vascular inva-
sion, no myometrial invasion or no lymphatic metastasis, underscoring the fact that SHP2 is a better prognostic factor than these clinico-
pathological prognostic factors for EC. Mean-
while, ROC curve comparison further confirmed that SHP2 combined with other clinicopatholog-
ical factors had highest specificity and sensitivi-
ty of prognosis.

EGFR is over-expressed in a large number of tumors and is one of the best characterized oncogenic targets. Our previous study [30] showed the expression of EGFR was correlated with surgical stage and histologic grade in EC.

Figure 6. Expressions of EGFR protein in endometrial samples. Original magnification×100. A: No EGFR expression in normal endometrium, defined as negative staining. B: EGFR expression in endometrial cancer grade 1, defined as positive staining. C: EGFR expression in endometrial cancer grade 2, defined as strong positive staining. D: EGFR expression in endometrial cancer grade 3, defined as strong positive staining.
as well as progression and prognosis. Concha-Benavente et al. [31] reported that EGFR mediated tumor immunoescape by inhibiting the activation of STAT1 and promoting that of STAT3, while SHP2 is responsible for the dephosphorylation of active STAT1 in this process. A fraction of SHP2 was found to be sequestered at the plasma membrane in lung cancer cells with EGFR mutation [19], and inhibition of the SHP2 PTP activity impaired mutant EGFR signaling and suppressed EGFR mutant-induced lung adenocarcinoma [32]. SHP2 expression was also reported to be associated with HER2 expression in breast cancer [33]. Inhibition of SHP2 in HER2-positive breast cancer cells abrogated survival signaling, resulting in a conversion to a normal breast epithelial phenotype [13]. In our study, SHP2 showed a positive correlation with expression of EGFR. Although we have known EGF stimulates SHP2 activation, it is not clear whether SHP2 is active in EC cells harboring EGFR and whether SHP2 is important for EGFR to drive EC. So further work, such as molecular mechanism with EGFR should be performed.

In summary, we found SHP2 expression increased in EC, and associated with tumor grade, lymph node metastasis and myometrial invasion. SHP2 expression is associated with EFGR. SHP2 is an independent negative prognostic factor for overall survival in EC, and may become a potential target for EC.

Disclosure of conflict of interest

None.

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