Expression of Phosphatase of Regenerating Liver 1 and 3 mRNA in Esophageal Squamous Cell Carcinoma

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Protein tyrosine phosphatases, as a subgroup of the protein phosphatase superfamily, play key roles in regulating functions of diverse proteins that control numerous essential events in eukaryotes, such as transcriptional regulation, apoptosis, cell cycle progression, protein degradation, and protein trafficking.1–3 Phosphatase of regenerating liver (PRL) is a new class of the protein tyrosine phosphatase family and consists of 3 members so far, PRL-1, PRL-2, and PRL-3.4,5 Although PRLs normally are expressed in the skeletal muscle and brain at high levels, in the heart at moderate levels, and in many other tissues at low level,5–7 aberrant expression of PRLs has been identified in a variety of cancer cell lines and tissues, especially in metastatic colorectal cancer.8–11 Preliminary experiments have demonstrated that inhibition of endogenous PRL in cancerous cells can abrogate cell motility and ability to metastasize in a mouse model.11 These findings strongly suggest PRLs’ potential to serve as biomarkers and therapeutic targets in cancer.

Esophageal carcinoma is the sixth most common malignant tumor worldwide.14 The incidence of esophageal carcinoma is very high in China.15 Effective treatment depends on early diagnosis, with which more than 90% of the patients can survive 5 to 10 years.16 Deep invasion and metastasis remain the leading causes of death for esophageal carcinoma patients. Most esophageal carcinoma in China is squamous cell carcinoma, different from predominantly adenocarcinoma in western countries. Nutrition, tobacco, and alcohol are believed to be major causes of esophageal squamous cell carcinoma (ESCC), whereas Barrett esophagus is considered a key precancerous lesion with a strong association with the development of dysplasia and subsequent esophageal adenocarcinoma.17

Overexpression of PRL-3 messenger RNA (mRNA) has been identified in several human cancers including colorectal, gastric, ovarian, breast, and hepatic cancers as compared with their normal tissues.10–12,15,18 Only a few cases of metastatic pancreatic and esophageal cancers were examined for PRL-3 expression.19 In this study, we examined expression of PRL-1 and PRL-3 mRNAs with reverse transcriptase–polymerase chain reaction (RT-PCR) in 40 cases of ESCC and analyzed their association with clinicopathologic parameters.

Materials and Methods

Tumor Samples

Forty cases of radical esophagectomy specimens were collected at Anyang Tumor Hospital, Henan, China. Fresh tissues were taken from tumor and resection margin (for normal esophageal mucosa). Most tumors (n = 28) were located at the middle one third and the rest were located at the upper (n = 4) and lower (n = 8) one third of the esophagus. The diagnoses of ESCC were confirmed histologically for all the tumors. Tumor classification and clinical stage were based on World Health Organization criteria.20 Of 40 ESCCs, 10, 15, and 15 were well, moderately, and poorly differentiated, respectively. Nineteen cases were stage IIA, 7 were stage IIB, and 14 were stage III.

In every case, 6 to 21 (mean, 10.5) lymph nodes were collected from paraesophageal, left gastric, right cardiac, mediastinal, and lower neck sites. Metastasis to lymph nodes was identified in 21 cases, mostly in left gastric and/or paraesophageal lymph nodes (n = 17).
The patients consisted of 26 men and 14 women and had an age range of 33 to 77 years with a mean age of 60 years. None of the patients received any type of therapy prior to surgery. Informed consent was obtained from all of the patients. The study was approved by the institutional review board.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was isolated from cancer and normal esophageal mucosa from all the cases with TRIZOL reagent (Invitrogen, Carlsbad, Calif) and reverse-transcribed with a One Step RNA PCR kit (AMV) (TaKaRa, Dalian, China). Briefly, 5 μL of total RNA was reverse-transcribed at 50°C and amplified for 35 cycles with an annealing temperature of 47°C for PRL-1 and 50°C for PRL-3 for 1.5 minutes. Primer sequences for PRL-1 were 5'-TACTGGATTGAAGAATTGCTGCT-3' (forward) and 5'-ACAA-CAACCAGGTTCTCAGA-3' (reverse) and for PRL-3 were 5'-ATGGCTCGGATGACCGCCCG-3' (forward) and 5'-CTACA-TAACCGACCGGGTC-3' (reverse). The housekeeping gene β-actin was used as an internal control. RT-PCR was performed in triplicate for all the experiments with the negative control in which no template (total RNA) was added. RT-PCR products underwent electrophoresis in 2% agarose gel containing ethidium bromide. The corresponding DNA bands were scanned and semi-quantitatively analyzed with an image analysis system (Stratagene, La Jolla, Calif). The units for PRL-1 and PRL-3 complementary DNA (cDNA) as well as β-actin bands were used to represent the levels of their mRNA expression after normalization to β-actin expression.

Statistical Analysis

All data were analyzed with SPSS version 11.0 statistical package (Shengce Software Ltd, Beijing, China). The differences in positive rates and means were analyzed by χ² and t test, respectively. The relationship of 2 variables was analyzed by correlation analysis. P values less than .05 were defined as statistically significant.

RESULTS

Electrophoresis of RT-PCR products demonstrated amplified cDNA fragments at expected size, 490 base pair for PRL-1, 510 base pair for PRL-3, and 330 base pair for β-actin (Figure 1, A; Figure 2, A). The frequency of PRL-1 mRNA expression was observed in 25 (62.5%) of 40 cases of ESCC, higher than that in normal esophageal mucosa (25%; 10/40) (Figure 1, B; Table 1). The difference was significant (P = .001). PRL-3 mRNA was expressed in a similar pattern but in fewer cases, 32.5% (13/40) in ESCC and 10% (4/40) in normal esophageal mucosa (Figure 2, B). The difference was also significant (P = .01). Of 21 cases of ESCC with lymph node metastasis, 17 (81%) cases expressed PRL-1 mRNA, more frequently than ESCC without metastasis (42.1%; 8/19) (Figure 1, B; Table 1). The difference was significant (P = .01). PRL-3 mRNA showed a similar pattern of expression, 47.6% (10/21) in ESCC with lymph node metastasis but only 15.8% (3/19) in ESCC without metastasis (Figure 2, B; Table 1; P = .03).

Association of PRL-1 mRNA expression with clinicopathologic parameters was analyzed. PRL-1 mRNA was much more frequently expressed in the tumors of stages IIIB and III than in those of stage IIA with significant differences (P = .04; P = .03; Table 2). PRL-3 mRNA expression also showed significantly higher frequency in tumors of stage IIIB, but not stage III, than IIA (P = .03; Table 2). PRL-1 and PRL-3 mRNA was expressed in similar frequencies in ESCC at stages IIB and III (Table 2). Frequency
of PRL-1 and PRL-3 mRNA expression did not show correlation with tumor location, tumor differentiation, patient's sex, and age (Table 2).

Semi-quantitative analyses showed the level of PRL-1 mRNA expression was similar in normal esophageal mucosa and ESCC (1.00 ± 0.05 vs 1.06 ± 0.10); so was the level of PRL-3 mRNA (0.81 ± 0.11 vs 0.87 ± 0.06). However, the level of PRL-1 mRNA expression was significantly higher in ESCC with lymph node metastasis (1.106 ± 0.08) than in ESCC without lymph node metastasis (0.98 ± 0.09; P = .04). Such significant difference was also identified in PRL-3 mRNA levels (0.92 ± 0.05 vs 0.78 ± 0.02; P = .04; Figure 3, A and B).

Among the clinicopathologic parameters, the level of PRL mRNA expression was correlated with the stage. PRL-1 mRNA level was much higher in ESCC stage IIB (1.08 ± 0.07) and stage III (1.12 ± 0.10) than in stage IIA (0.98 ± 0.09) with significant differences (P = .04; P = .03). PRL-3 mRNA level was also higher in ESCC stage IIB (0.91 ± 0.06) and stage III (0.93 ± 0.05) than in stage IIA (0.78 ± 0.02) with significant differences (P = .04; P = .04). The levels of PRL-1 and PRL-3 mRNA expression were not correlated with tumor differentiation, tumor location, patient's sex, and age (data not shown).

To explore the correlation of PRL-1 and PRL-3 mRNA expression in ESCC, the frequencies of their mRNA expression were compared. Analysis showed a positive correlation between PRL-1 and PRL-3 mRNA expression in ESCC (Table 3) and PRL-1 mRNA was more often expressed than PRL-3 mRNA in ESCC with significant difference (P = .004). Interestingly, 10 of 11 cases of ESCC with expression of both PRL-1 and PRL-3 mRNAs had lymph node metastasis.

**COMMENT**

Phosphatase of regenerating liver was originally identified as an immediate early gene after partial hepatectomy (thus named phosphatase of regenerating liver), but it was soon found to associate with human tumors. Phosphatases of regenerating liver were frequently expressed in a variety of tumor cell lines. Studies on expression of PRL in cancer tissues are also emerging. Significantly higher frequency of PRL-3 expression has been observed in several human cancers including colorectal, gastric, ovarian, hepatic, and breast cancers as compared with their normal tissues. Only 2 metastatic esophageal cancers were mentioned in 1 study on PRL, and it was not clarified as squamous cell carcinoma or adenocarcinoma. In this study, we examined expression of PRL-1 and PRL-3 mRNAs in 40 cases of ESCC with RT-PCR. The frequencies of phosphatase of regenerating liver (PRL) 3 mRNA expression in benign and malignant esophageal tissues. A, Agarose gel image of reverse transcriptase–polymerase chain reaction products. bp indicates base pair; M, molecular marker; N, normal esophageal mucosa; and T, tumor. B, Bar graph of PRL-3 mRNA expression in 40 cases of esophageal squamous cell carcinoma (ESCC). LN indicates ESC without lymph node metastases; LN+, ESCC with lymph node metastases.
Table 2. Association of Phosphatase of Regenerating Liver (PRL) 1 and PRL-3 mRNA Expression Frequency With Clinicopathologic Parameters

<table>
<thead>
<tr>
<th></th>
<th>Total Cases, No.</th>
<th>PRL-1*, No. (%)</th>
<th>P Values</th>
<th>PRL-3*, No. (%)</th>
<th>P Values</th>
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<td><strong>Stage</strong></td>
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<tr>
<td>IIA</td>
<td>19</td>
<td>8 (42.1)</td>
<td>.04</td>
<td>3 (15.8)</td>
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<tr>
<td>IIB</td>
<td>7</td>
<td>6 (85.7)</td>
<td>.03, .75*</td>
<td>4 (57.1)</td>
<td>.09, .62*</td>
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<td>III</td>
<td>14</td>
<td>11 (78.6)</td>
<td>.09</td>
<td>6 (42.9)</td>
<td>.71, .47*</td>
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<td><strong>Tumor differentiation</strong></td>
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<td></td>
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<tr>
<td>Well</td>
<td>10</td>
<td>6 (60.0)</td>
<td>.04</td>
<td>2 (20.0)</td>
<td>.03</td>
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<td>Moderate</td>
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<td>8 (53.3)</td>
<td>.74</td>
<td>6 (40.0)</td>
<td>.29</td>
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<td>Poor</td>
<td>15</td>
<td>11 (73.3)</td>
<td>.48, .26*</td>
<td>5 (30.0)</td>
<td>.71, .47*</td>
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<td><strong>Tumor location in the esophagus</strong></td>
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<td>Upper one third</td>
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<td>2 (50.0)</td>
<td>.47</td>
<td>2 (50.0)</td>
<td>.48</td>
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<td>Middle one third</td>
<td>28</td>
<td>18 (64.3)</td>
<td>.47</td>
<td>9 (32.0)</td>
<td>.48</td>
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<td>Lower one third</td>
<td>8</td>
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<td>.&gt;99, .47*</td>
<td>2 (25.0)</td>
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<td>61–70</td>
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<td>5 (41.7)</td>
<td>.98</td>
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<td>&gt;70</td>
<td>6</td>
<td>3 (50.0)</td>
<td>.84, .25*, .&gt;99†</td>
<td>1 (14.0)</td>
<td>.29*, .34†</td>
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</table>

* Compared with the second group.
† Compared with the third group.

Figure 3. Levels of phosphatase of regenerating liver (PRL) 1 and PRL-3 mRNA expression in esophageal squamous cell carcinoma (ESCC) with and without lymph node metastasis. A, Agarose gel image of reverse transcriptase–polymerase chain reaction products. bp indicates base pair; M, molecular marker; LN−, ESCC without lymph node metastases; and LN+, ESCC with lymph node metastases. B, Semiquantitative evaluation of PRL-1 and PRL-3 mRNA expression levels in 40 cases of ESCC.
**Table 3. Correlation of Phosphatase of Regenerating Liver (PRL) 1 and PRL-3 mRNAs in Esophageal Squamous Cell Carcinoma**

<table>
<thead>
<tr>
<th>PRL-1</th>
<th>PRL-3</th>
<th>+</th>
<th>-</th>
<th>Total</th>
<th>$r_s$</th>
<th>$P$</th>
<th>$P^+$</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>13</td>
<td>0.317</td>
<td>.04</td>
<td>.004</td>
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* $r_s$ indicates Pearson R Spearman correlation; $P$, significant positive correlation; and $P^+$, significant difference in frequency of PRL-1 and PRL-3 mRNA expression, calculated by McNemar test.

Phosphatases of regenerating liver do have preferential expression in normal tissues, such as high level of PRL-1 in the brain and moderate level of PRL-3 in the heart.\textsuperscript{5,6} PRL-1 and PRL-2, rather than PRL-3, are also detected in many other tissues at low level.\textsuperscript{5,7} Most studies on PRL expression in cancers focus on PRL-3. There are only a few reports for parallel studies on 2 or 3 PRLs.\textsuperscript{17,26} Zeng at al\textsuperscript{26} compared functions of PRL-1 and PRL-3 in Chinese hamster ovary cells and found both of them, after transfection, were able to promote the migration and invasive activity of Chinese hamster ovary cells. Interestingly, Zeng et al\textsuperscript{26} also found that both PRL-1 and PRL-3 can promote the metastasis of Chinese hamster ovary cells in nude mice. However, Radke et al\textsuperscript{17} found that only PRL-3, but not PRL-1 or PRL-2, mRNA is expressed in significantly higher frequency in malignant rather than benign breast tissue. Our parallel studies on PRL-1 and PRL-3 showed a positive correlation of their mRNA expressions in ESCC. However, PRL-1 mRNA was much more frequently expressed than PRL-3 in ESCC. This phenomenon may result from the different expression patterns of PRL-1 and PRL-3 because PRL-1 is preferentially expressed in several digestive epithelial tissues including the esophagus during development.\textsuperscript{20} PRL-3 mRNA expression seems more specific, although less sensitive, than PRL-1 for ESCC with lymph node metastasis (77% vs 68%), whereas the paired PRL-1 and PRL-3 will serve as the best predictor for ESCC to metastasize because 10 of 11 cases of ESCC with expression of both PRL-1 and PRL-3 mRNAs have lymph node metastases.

Tumor metastasis is responsible for most cancer deaths.\textsuperscript{27,28} The close association of PRL-3 with tumor metastasis has intrigued people to explore its potential role as the therapeutic target for cancers. The effectiveness and feasibility of such an approach have been proven experimentally. The expression of the catalytically inactive PRL-3 mutant significantly reduced the cell migratory capability.\textsuperscript{10} The higher migratory ability in PRL-3-overexpressed cells could be reversed by specific antisense oligodeoxynucleotide and the phosphatase inhibitors sodium orthovanadate or potassium bisperoxo oxovanadate V.\textsuperscript{10} PRL-3-specific knockdown using small interfering RNA severely impaired the growth of ovarian cancer cells\textsuperscript{11} and abrogated motility (in vitro) and hepatic colonization (in vivo) of human colon cancer DLD-1 cells.\textsuperscript{12} The antiprotozoal drug, pentamidine, is an inhibitor of PRLs and can inactivate exogenous PRLs with a long effective duration (>24 hours) after a pulse cell treatment at its therapeutic dose. It also inhibits growth of human cancer cell lines expressing endogenous PRLs. The growth of WM9 human melanoma tumors could also be markedly inhibited by pentamidine at a tolerable dose in nude mice with the induction of tumor cell necrosis.\textsuperscript{29} As high as 91% frequency of PRL-1 mRNA expression in ESCC with lymph node metastasis identified in this study will provide convincing evidence for exploring such a possibility to prevent and treat metastasis of ESCC in the future.

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**References**