Thymidine phosphorylase expression in progression of cervical cancer: correlation with microvessel count, proliferating cell nuclear antigen, and apoptosis

Ritsuto Fujiwaki, Kohkichi Hata, Kohji Iida, Yoshinobu Maede, Mikio Koike, Kohji Miyazaki

Abstract

Aims—To determine how epithelial and stromal thymidine phosphorylase expression affects angiogenesis, rapid tumour growth, and decreased apoptotic activity in cervical cancer at varying stages of progression.

Methods—Epithelial and stromal thymidine phosphorylase expression, the microvessel count (reflected by factor VIII related antigen), and proliferating cell nuclear antigen (PCNA) were assessed immunohistochemically in 25 specimens of normal cervical epithelium, 35 of carcinoma in situ (CIS), 34 of microinvasive carcinoma, and 34 of invasive cervical squamous cell carcinoma. Apoptosis was evaluated by the terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labelling (TUNEL) method. The relation of epithelial and stromal thymidine phosphorylase expression to microvessel count, PCNA index, and apoptotic index was examined.

Results—Epithelial and stromal thymidine phosphorylase expression progressively increased along a continuum from normal epithelium to invasive squamous cell carcinoma. Epithelial and stromal thymidine phosphorylase expression showed a significant positive correlation with microvessel counts. Within each histological stage, CIS cases with high stromal thymidine phosphorylase expression, invasive squamous cell carcinoma cases with high epithelial thymidine phosphorylase expression, and microinvasive carcinoma cases with high epithelial thymidine phosphorylase expression and microinvasive carcinoma cases with high stromal thymidine phosphorylase expression were associated with a significantly higher microvessel count. High epithelial thymidine phosphorylase expression was associated with a significantly higher PCNA index in CIS and microinvasive carcinoma, but not in invasive squamous cell carcinoma. No significant correlation was seen between apoptotic index and either epithelial or stromal thymidine phosphorylase expression or microvessel count.

Conclusions—Epithelial and stromal thymidine phosphorylase expression may combine to promote angiogenesis during progression of cervical cancer, and epithelial thymidine phosphorylase expression may stimulate tumour cell proliferation in the early stages.

Keywords: thymidine phosphorylase; microvessel count; proliferating cell nuclear antigen; cervical cancer

The importance of angiogenesis for the growth of solid tumours is well recognised. Tumours are not usually angiogenic early in their development, when they remain limited to a small volume of a few cubic millimetres. Some tumour cells then acquire an angiogenic phenotype and elicit growth of new capillaries to support tumour growth. Angiogenesis is induced by many factors produced by tumour cells as well as by non-malignant stromal cells that infiltrate the tumour. Among these angiogenic factors, thymidine phosphorylase, which is identical to platelet derived endothelial cell growth factor, has been shown to be important in angiogenesis. Thymidine phosphorylase itself is chemotactic to endothelial cells in vitro and is angiogenic in vivo. One of its degradation products, 2-deoxy-D-ribose, is also angiogenic. Immunohistochemically detectable thymidine phosphorylase expression has been found in several studies in response to a variety of human malignant tumour cells, and it has been concluded that thymidine phosphorylase is an important regulator of tumour angiogenesis. In addition to tumour cells, thymidine phosphorylase expression by stromal cells such as fibroblasts, macrophages, and lymphocytes has been implicated in tumour neovascularisation in invasive colonic, gastric, and lung cancers.

An increase in microvessel count has been found in early malignant conditions. It precedes the appearance of frank invasion, suggesting that activation of angiogenesis is an essential early event in tumour development. How thymidine phosphorylase affects angiogenesis in such early cancer stages as carcinoma in situ (CIS) and microinvasive carcinoma has not been adequately evaluated.

Thymidine phosphorylase has been related to tumour growth and apoptotic activity in recent experimental studies. Overexpression of thymidine phosphorylase has been shown to increase tumour growth in vivo in a nude mouse model. Takebayashi et al have shown that KPE-3 cells transfected with thymidine phosphorylase cDNA were resistant to apopto-
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marker for proliferative activity,15 and apoptotic-proliferating cell nuclear antigen (PCNA) as a cervix. We investigated the relation of thymidine phosphorylase expression in specimens of normal epithelial and stromal thymidine phosphorylase from the uterine cervix is a representative human neoplasm with a clear.

during human tumour development it is not thymidine phosphorylase a ect these factors involved in tumorigenesis through proliferative and antiapoptotic activities. However, whether thymidine phosphorylase affects these factors during human tumour development it is not clear.

Squamous cell carcinoma of the uterine cervix is a representative human neoplasm with a well known natural history of tumour development. In the present study, we examined epithelial and stromal thymidine phosphorylase expression in specimens of normal epithelium, CIS, microinvasive carcinoma, and invasive squamous cell carcinoma from the uterine cervix. We investigated the relation of thymidine phosphorylase to microvessel count, proliferating cell nuclear antigen (PCNA) as a marker for proliferative activity,16 and apoptosis.

Methods

CASE SELECTION

We selected 128 specimens from the surgical tissue archives at Shimane Medical University. The operations were either conisations or hysterectomy, performed between September 1984 and November 1997, in which the uterine cervix was processed completely for histological assessment. Tissue had been fixed routinely in 10% formalin and embedded in paraffin. Cases were randomly selected, mostly by availability of blocks or clinicopathological data. After initial review of all available haematoxylin and eosin stained slides by one investigator (RF), we selected for each case a representative paraffin embedded block, in which maximum depth of stromal invasion and/or maximum tumour width were present. Cervical cancers were graded according to the International Federation of Gynaecology and Obstetrics (FIGO),16 including 35 cases of CIS, 34 cases of microinvasive carcinoma (maximum depth of stromal invasion 5 mm; maximum width 7 mm), and 34 cases of invasive squamous cell carcinoma (clinical stage Ia to IIIa). Twenty five normal cervical specimens, obtained from women who underwent hysterectomy for benign non-cervical conditions, were examined as controls.

IMMUNOHISTOCHEMISTRY AND EVALUATION OF IMMUNOSTAINING

Immunohistochemical analysis was performed on formalin fixed, paraffin embedded tissue sections (4 µm) using a labelled streptavidin–biotin method (LSAB kit; Dako Japan). Briefly, the dewaxed, rehydrated sections were treated with 0.3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase absorption of antisera. Primary antibodies were added to the slides and incubated for two hours at room temperature and then washed with PBS.

Primary antibodies used were a mouse monoclonal antibody 654-1 (Nippon Roche Research Centre, Kamakura, Japan) at a 1:100 dilution for thymidine phosphorylase, a rabbit polyclonal antibody F8/86 (Dako) at a 1:40 dilution for factor VIII, and a mouse monoclonal antibody PC 10 (Dako) at a 1:40 for PCNA. Thymidine phosphorylase antibody 654-1 is a monoclonal antibody prepared using an enzyme purified from human colorectal carcinoma xenograft HCT 116. The specificity of this antibody has been determined by western blot analysis, and the protein levels measured by this antibody have been correlated with enzyme activity by enzyme linked immunosorbent assay.17

Sections were then incubated with biotin labelled secondary antibody for 20 minutes and washed with PBS. Peroxidase conjugated streptavidin was added for 20 minutes and then washed with PBS. Finally, bound antibody complexes were stained for 10 minutes with 0.05% diaminobenzidine and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.6). Sections were then counterstained with methyl green, dehydrated, and mounted. A normal liver section was used as a positive control, as Kupffer cells are known to express a high level of thymidine phosphorylase. To assess the immunospecificity of each antibody, normal rabbit serum (negative control) was used instead of primary antibodies.

Thymidine phosphorylase positivity was defined as unequivocal staining in the cytoplasm or nucleus. The intensity of thymidine phosphorylase staining in epithelial cells and stromal cells was graded on an arbitrary scale of 0 to 3+: 0, no positive cells; 1+, slight staining; 2+, moderate staining; 3+, strong staining.7 Microvessels were counted in the tumour according to the criteria of Guidi et al.18 Briefly, only specimens with adequate stroma (three or more × 400 fields consisting of > 90% stroma) were evaluated. Microvessels were identified by factor VIII positive endothelial cells, with or without discrete lumina. Results are expressed as the largest number of microvessels present within a single × 400 field. The PCNA index was determined by counting at least 1000 cancer cells, and was expressed as a percentage of positive nuclear cells.

APOPTOTIC INDEX BY TUNEL METHOD

Apoptotic cells were identified by a terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine 5-triphosphate biotin nick end labelling (TUNEL) method, using the ApoTag in situ detection kit (Oncor). Briefly, dewaxed and rehydrated sections were incubated in proteinase K for 10 minutes, and were treated with 0.3% hydrogen peroxide in methanol for 30 minutes. After adding equilibration buffer for 10 minutes, terminal deoxynucleotidyl transferase was pipetted onto
sections which then were incubated at 37°C for one hour. The reaction was stopped by incubating sections in stop buffer for 10 minutes. Anti-digoxigenin–peroxidase was added to the slides and incubated for 30 minutes. Slides were stained with diaminobenzine for four minutes and counterstained with methyl green. A specimen of thyroid gland known to be positive for apoptotic cells was used as positive control, and substitution of distilled water for TdT was used as a negative control.

The apoptotic index was determined by light microscopy according to the criteria of Lu and Tanigawa. We identified TUNEL positive tumour cells with morphological characteristics of apoptosis such as chromatin condensation, nucleolar disintegration, formation of crescentic caps of condensed chromatin at the nuclear periphery, and nuclear residues with intense staining. Positively staining cells in the stroma, lumen, and necrotic areas were excluded. The apoptotic index was determined by evaluating at least 2000 tumour cells to determine the percentage of TUNEL positive cells.

Evaluation of immunostaining and the apoptotic index was determined by two investigators simultaneously (RF and KH). They had no knowledge of the patients’ clinicopathological data. Disputed assessments for thymidine phosphorylase expression were subjected to a further blind assessment by a third investigator (YM). Cases in which interobserver differences of microvessel count, PCNA index, and apoptotic index exceeded 10% were subjected to joint re-evaluation.

**STATISTICAL ANALYSIS**

The Mann–Whitney U test (for comparison of two groups) or Kruskal–Wallis one way analysis of variance and Bonferroni’s multiple comparison test (for comparison of three groups) was used to evaluate non-parametric numeric data. Fisher’s exact test or χ² test was used for comparisons of categorical data. The correlation between different variables was done with the Spearman rank correlation test. A probability (p) value of < 0.05 was considered statistically significant.

**Figure 1** Various patterns of immunohistochemical staining in normal uterine cervix and cervical cancer using an antibody to thymidine phosphorylase. (A) Superficial layers of normal epithelium show weak thymidine phosphorylase expression, but the basal cell compartment of the epithelium and the stroma show no immunoreactivity (×35). (B) Strong epithelial thymidine phosphorylase expression is not accompanied by stromal thymidine phosphorylase expression in a case of microinvasive carcinoma (×35). (C) No epithelial thymidine phosphorylase expression but strong stromal thymidine phosphorylase expression is seen in a case of carcinoma in situ (×35). (D) Strong epithelial expression and strong stromal expression is seen in a case of invasive squamous cell carcinoma (×28). The counterstain used is methyl green.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Epithelial and stromal expression of thymidine phosphorylase (TP) in each histological stage</th>
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<tbody>
<tr>
<td>Histological stage</td>
<td>High epithelial TP expression</td>
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<tr>
<td>Normal</td>
<td>25</td>
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<tr>
<td>CIS</td>
<td>35</td>
</tr>
<tr>
<td>MIC</td>
<td>34</td>
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<td>Invasive SCC</td>
<td>34</td>
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</table>

a–b, p < 0.01; a–c, e–f, e–g, p < 0.001; a–d, e–h, p < 0.0001 (Fisher’s exact test). CIS, carcinoma in situ; MIC, microinvasive carcinoma; SCC, squamous cell carcinoma.
expression, as this staining intensity was seen in less than 5% of normal cervixes. In both epithelium and stroma, high thymidine phosphorylase expression was significantly more common in CIS, microinvasive carcinoma, and invasive squamous cell carcinoma than in normal specimens (table 1).

RELATION BETWEEN THYMIDINE PHOSPHORYLASE EXPRESSION AND MICROVESSEL COUNT

The highest microvessel count was noted in a narrow zone immediately underlying the neoplastic epithelium. Mean (SD) microvessel counts were 22.7 (7.8), 33.4 (13.0), 33.6 (16.9), and 39.7 (16.2), for normal epithelium, CIS, microinvasive carcinoma, and invasive squamous cell carcinoma, respectively. There was a significant correlation between histological stage of progression and microvessel count (p = 0.36, p < 0.001). Epithelial and stromal thymidine phosphorylase expression correlated positively with microvessel counts (p = 0.25, p < 0.05, and p = 0.35, p < 0.001, respectively). We examined the relation between epithelial or stromal thymidine phosphorylase expression and microvessel count for each histological stage. CIS cases showing high stromal thymidine phosphorylase expression (score > 2+) and invasive squamous cell carcinoma cases showing high epithelial thymidine phosphorylase expression (score > 2+) had a significantly higher microvessel count than other cases (p < 0.05, and p < 0.05, respectively; table 2). In microinvasive carcinoma, cases with both high epithelial and high stromal thymidine phosphorylase expression showed significantly greater microvessel count (p < 0.05).

RESULTS

THYMIDINE PHOSPHORYLASE EXPRESSION

The basal cell compartment of normal cervical epithelium was usually not immunoreactive for thymidine phosphorylase; only one specimen (4%) showed a moderately intense reaction. In superficial layers of the epithelium, weak expression could be detected in some specimens (fig 1A).

Thymidine phosphorylase expression was also very low in normal stromal tissue, and only one specimen showing moderate reactivity. In contrast, significant immunoreactivity was observed in CIS, microinvasive carcinoma, and invasive squamous cell carcinoma (fig 1B, C, and D). Epithelial thymidine phosphorylase expression was distributed diffusely through the full thickness of the neoplastic epithelium. The usual pattern was cytoplasmic, but nuclear staining was also observed. In addition, endothelial cells, fibroblasts, and tumour associated inflammatory cells, appearing to be macrophages or lymphocytes, also showed immunoreactivity. Immunoreactivity of thymidine phosphorylase was rare in histologically benign epithelial and stromal tissues adjacent to the neoplastic lesions. A progressive increase in score of epithelial and stromal thymidine phosphorylase expression was seen in a continuum from normal tissue to invasive squamous cell carcinoma (p = 0.43, p < 0.0001; p = 0.42, p < 0.0001, respectively). Epithelial thymidine phosphorylase expression score correlated significantly with stromal thymidine phosphorylase expression (p = 0.37, p < 0.0001). A tumour with a score of at least 2+ was considered to show high epithelial or stromal thymidine phosphorylase

<table>
<thead>
<tr>
<th>PCNA index</th>
<th>CIS (n=35)</th>
<th>MFC (n=34)</th>
<th>Invasive SCC (n=34)</th>
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<tbody>
<tr>
<td>Epithelial TP expression</td>
<td></td>
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<tr>
<td>Low (&lt;2+)</td>
<td>38.4 (19.6)</td>
<td>37.5 (21.8)</td>
<td>57.3 (22.2)</td>
</tr>
<tr>
<td>High (≥2+)</td>
<td>55.0 (24.2)</td>
<td>53.8 (21.3)</td>
<td>64.5 (21.9)</td>
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<td>Stromal TP expression</td>
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<tr>
<td>Low (&lt;2+)</td>
<td>45.0 (20.0)</td>
<td>42.7 (19.1)</td>
<td>61.0 (17.2)</td>
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<tr>
<td>High (≥2+)</td>
<td>44.1 (26.0)</td>
<td>49.2 (28.1)</td>
<td>62.0 (24.7)</td>
</tr>
</tbody>
</table>

Values are mean (SD).

a–b, c–d, p < 0.05 (Mann–Whitney U test); c–d, p < 0.05 (Bonferroni multiple comparison test).

CIS, carcinoma in situ; MFC, microinvasive carcinoma; SCC, squamous cell carcinoma.

EPITHELIAL AND STROMAL THYMIDINE PHOSPHORYLASE EXPRESSION, MICROVESSEL COUNT, AND PCNA INDEX

Mean (SD) PCNA indices were 10 (14.3), 44.2 (24.3), 45.1 (22.9), and 61.7 (22.0) in normal epithelium, CIS, microinvasive carcinoma, and invasive squamous cell carcinoma, respectively. Significant positive correlations were observed between epithelial or stromal thymidine phosphorylase expression and the PCNA index (p = 0.44, p < 0.0001, and p = 0.35, p < 0.001). Microvessel count tended to correlate with the PCNA index, falling short of statistical significance (p = 0.07). In analyses within histological stages, CIS and microinvasive carcinoma cases with high epithelial thymidine phosphorylase expression had significantly higher PCNA indices than others (p < 0.05 and p < 0.05, respectively); no such difference was observed among cases of invasive squamous cell carcinoma (table 3). Neither stromal thymidine phosphorylase expression nor microvessel count correlated with the PCNA index in any histological stage.

EPITHELIAL AND STROMAL THYMIDINE PHOSPHORYLASE EXPRESSION, MICROVESSEL COUNT, AND APOPTOTIC INDEX

Mean (SD) apoptotic indices were 0.29 (0.19), 0.47 (0.32), 0.71 (0.53), and 1.47 (1.14) in normal epithelium, CIS, microinvasive carci-
Table 4 Relation between thymidine phosphorylase (TP) expression and apoptotic index in each histological stage

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<tr>
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<th>Apoptotic index</th>
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<tr>
<td></td>
<td>CIS (n=35)</td>
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<tr>
<td>Epithelial TP expression</td>
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<tr>
<td>Low (&lt;2+)</td>
<td>0.45 (0.36)</td>
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<tr>
<td>High (≥2+)</td>
<td>0.51 (0.24)</td>
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<tr>
<td>Stromal TP expression</td>
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<tr>
<td>Low (&lt;2+)</td>
<td>0.53 (0.35)</td>
</tr>
<tr>
<td>High (≥2+)</td>
<td>0.39 (0.24)</td>
</tr>
</tbody>
</table>

Values are mean (SD). CIS, carcinoma in situ; MIC, microinvasive carcinoma; SCC, squamous cell carcinoma.

Discussion

Our data show that epithelial and stromal thymidine phosphorylase expression and microvessel count were significantly greater in CIS than in normal cervical mucosa. These characteristics progressively increased from CIS to invasive squamous cell carcinoma, suggesting that heightened thymidine phosphorylase expression and angiogenesis occur as part of the preinvasive process, intensifying during the course of tumour development. Epithelial and stromal thymidine phosphorylase expression was positively correlated with microvessel count. Interestingly, specific aspects of apparent thymidine phosphorylase interactions with angiogenesis were not uniform within the histological stages. High stromal thymidine phosphorylase expression in CIS cases, high thymidine phosphorylase expression in both epithelium and stroma in microinvasive carcinoma cases, and high epithelial thymidine phosphorylase expression in invasive squamous cell carcinoma cases were associated with significantly higher microvessel count. The site responsible for stimulating angiogenesis by way of thymidine phosphorylase may shift from stromal cells to the neoplastic epithelial cells as the tumour progresses. These results suggest that thymidine phosphorylase may play an important role in angiogenesis, involving cooperative epithelial and stromal expression of the enzyme. However, conflicting findings have been reported, including the finding of no correlation between epithelial thymidine phosphorylase expression and microvessel count in invasive cervical cancer. While the reason for this discrepancy is not clear, the studies involved different specimens (squamous cell carcinomas vs squamous cell and adenocarcinomas), different immunohistochemical methods (with or without microwave antigen retrieval), and different methods of evaluating thymidine phosphorylase staining (staining intensity vs percentage of cells stained). Recently, Dobbs et al and other investigators reported an increase in microvessel counts from normal through to malignant tissues and showed a strong correlation between microvessel counts and vascular endothelial growth factor (VEGF) immune activity. Further investigations are necessary to determine the relation between VEGF and thymidine phosphorylase expression and the correlation between expression and microvessel count.

Tumour cell proliferative activity is closely related to tumour progression. PCNA protein, a marker of proliferative activity, is expressed in late G1-S phase. Increasing proliferative activity according to the PCNA index has been shown to occur as tumours progress from preinvasive lesions toward invasive cervical cancer. In the present study, epithelial and stromal thymidine phosphorylase expression correlated positively with the overall PCNA index in the specimen studied, but microvessel count did not. Additionally, CIS and microinvasive carcinoma cases with high epithelial thymidine phosphorylase expression had significantly greater PCNA indices than other cases. Our findings are consistent with reports that tumours formed by human carcinoma cells transfected with thymidine phosphorylase cDNA and then grafted into nude mice grew more rapidly than those arising from transplanted non-transfected control cells or from cells transfected with mutant cDNA without thymidine phosphorylase activity. Although it is not clear exactly how thymidine phosphorylase affects tumour growth, our results suggest that thymidine phosphorylase stimulates tumour proliferative activity in the early stage of cervical cancer. However, we found no correlation between thymidine phosphorylase expression and the PCNA index in invasive squamous cell carcinoma. Many influences including growth factors, activation of proto-oncogenes, and abnormalities of cell cycle regulators may supersede thymidine phosphorylase in effects on tumour proliferative activity in this last stage of progression.

Like tumour cell proliferation, apoptotic activity can affect tumour development. In agreement with our results, increased apoptotic activity has been associated with tumour progression in other studies of cervical cancer. Recent experiments have shown that KPE-3 cells transfected with thymidine phosphorylase cDNA were resistant to apoptosis induced by hypoxia. In clinical studies, Ueda et al have found that high thymidine phosphorylase expression correlated significantly with a low apoptotic index in invasive cervical cancer after preoperative chemotherapy. However, we found no correlation between thymidine phosphorylase expression and apoptotic index, either in specimens considered overall or in those analysed separately by histological stage. Some antineoplastic agents can induce apoptotic cell death: after preoperative chemotherapy, respective mean apoptotic indices of 12.4% and 7.6% were reported in two patients with complete and partial responses; these indices were much higher than in our cases. Such treatment related differences in apoptotic activity may...
have contributed to discrepancies between our results and those previously reported.

Although we determine PCNA index or apoptotic index by counting at least 1000 cancer cells, it may be difficult to study the effect of thymidine phosphorylase on cell proliferation or apoptosis without knowing the total number of cells being studied in the specimen. For example, if the number of cells undergoing proliferation or apoptosis in two specimens was the same, but one specimen contained twice the number of cells, then the proportion of cells undergoing proliferation or apoptosis would be different. However, our method has been used by several other investigators and we consider our result to be reliable.

In conclusion, it appears that epithelial and stromal thymidine phosphorylase expression cooperatively promotes angiogenesis during the progression of cervical cancer, and epithelial thymidine phosphorylase expression may stimulate tumour cell proliferation in the early stages. Thymidine phosphorylase may therefore play an important role in cervical tumorigenesis and specific inhibition of this enzyme should be considered in the treatment of cervical cancer.  

We thank Miss Taeko Yamada, Department of Obstetrics and Gynaecology, Shimane Medical University, for the management of the medical records.