Abstract. The aim of this study was to investigate the diagnostic value of tumor M2 pyruvate kinase (Tu-M2-PK) as a tumor marker in patients with pre-invasive (CIN), invasive (PCC) and recurrent (RCC) cervical cancer. Materials and Methods: Plasma samples were investigated from 125 patients, comprising 50 cases of CIN (I-III), 51 of PCC (FIGO I-IV) and 24 of RCC, before treatment. Tu-M2-PK levels were determined by using a quantitative sandwich enzyme immunoassay. Results: With the increase in disease severity from CIN to PCC to RCC, levels of Tu-M2-PK significantly increased (p<0.001). Levels of Tu-M2-PK significantly increased with respect to the FIGO stage (p<0.001) and had significantly higher values in node+ patients (p=0.028). There was no significant difference in Tu-M2-PK levels in CIN I-III patients (p=0.626). Patients with distant metastasis had significantly elevated levels of Tu-M2-PK (p<0.001). Conclusion: Tu-M2-PK can be used as a marker to differentiate between malignant and premalignant cervical lesions. In addition, the concentration of Tu-M2-PK correlates with the clinical stage of the disease.

Pyruvate kinase (PK) is a key enzyme of glycolysis. PK exists in several isoforms designated L, R, M1 and M2, and is responsible for the anaerobic production of ATP. During tumorogenesis (1-3), there is a change in the isoenzyme of PK with tissue-specific, tetrameric isoenzymes being replaced by the dimeric form M2-PK (4-6). For this reason, the dimeric form of M2-PK is called tumor M2 PK (Tu-M2-PK). Tu-M2-PK is released from tumor cells into the blood and is quantitatively detectable. This suggests that Tu-M2-PK can be used as a tumor marker.

The E7 oncoprotein of the human papilloma virus (HPV) type 16 binds directly to M2-PK, resulting in a transformation of the tetrameric into the dimeric form, as has been determined in ras-expressing normal rat kidney (NRK) cells. As a result, Tu-M2-PK is stabilized and large amounts of phospho-metabolites can accumulate, which can then serve as basic building blocks for biosynthetic processes within the framework of amplified tumor genesis (1-3).

In agreement with various immunohistochemical results, it has been shown that there is an increased concentration of Tu-M2-PK in the blood of patients with stomach or ...
colorectal carcinoma (7-12), pancreatic carcinoma (13-15), renal cell (16-22) and lung carcinoma (23-25), and malignant melanoma (26), as well as gynecological carcinomas such as ovarian (27), breast (28-31) and cervical carcinoma (32).

Tumor M2 pyruvate kinase is a non organ-specific marker that reflects the metabolic activity of a tumor. The lack of an optimal tumor marker for cervical carcinoma brought us to investigate Tu-M2-PK in pre-malignant and malignant lesions of the cervix uteri.

Materials and Methods

Patients. This study included 125 women presenting at the University Hospital Charité Berlin between 2002 and 2005 with histologically confirmed cervical lesions. Their mean age was 43 years (range, 19-92 years). All patients were staged following the guidelines of the International Federation of Gynecology and Obstetrics (FIGO). Fifty women had a precancerous lesion CIN I-III (group 1), 51 women were diagnosed with primary cervical cancer FIGO stage I-IV (Group 2) and 24 women suffered a relapse of cervical cancer (Group 3). Inclusion criteria for patient participation were the diagnosis of cervical cancer or a precancerous lesion, the age of 18 years or older and no former treatment for the actual stage of disease.

Plasma analysis. In all patients, blood samples were obtained by peripheral venous puncture prior to therapy. Samples were withdrawn into 10 ml EDTA tubes and centrifuged at 3620 rpm for 10 min. The supernatant plasma was removed and frozen in aliquots at 85°C until testing. All patients gave their written informed consent for the blood collection.

The concentration of Tu-M2-PK in the plasma was measured by a commercially available sandwich enzyme-linked immunosorbent assay (ScheBo®; Biotech AG Gießen, Germany) according to the manufacturer’s instructions. The monoclonal antibodies used are specific for Tu-M2-PK and do not react with other isoforms of PK. The cut-off value recommended by the manufacturer of 15 U/ml (limit of detection 5-100 U/ml given by manufacturer) corresponds to a specificity of 90% for a control group with diseases other than tumors (n=393).

The squamous cell carcinoma (SCC) antigen (SSC-AG) was determined in serum by the Central Institute for Laboratory Medicine and Pathobiocmetry of the Charité Berlin. The automated enzyme immune assay system ARCHITECT of Abbott Diagnostics (Hofheim-Wallau, Wiesbaden, Germany) was used. The reference value was an SCC-AG <1.5 μg/l as of age 18 years. The SCC-AG analysis involved 31 patients with accessible SCC data and an invasive cervical carcinoma.

Statistical analysis. Patient characteristics and clinical details were documented and tabulated in EXCEL. Statistical analysis was carried out using SPSS 13.0 software for Windows (licensed by Humbold University Berlin). Comparison of groups was carried out using non-parametric tests (Mann-Whitney and Kruskal-Wallis tests). Descriptive statistics of patient characteristics were determined using standard methods for the mean, median, standard deviation, as well as minimum and maximum values. The receiver-operator characteristics (ROC) curve was used for analysis of the results of Tu-M2-PK to determine cut-off values for the diagnosis of cervical carcinoma. The relationship between different variables was determined by the coefficient of correlation (r) based on the non-parametric Spearman rank correlation test. All reported significance was two-tailed at a level of 0.05.

Results

The mean Tu-M2-PK plasma concentration for the CIN group was below the cut-off value of 15 U/ml recommended by the manufacturer. In contrast, both the invasive cervix carcinoma and the relapse groups had a value above the cut-off. Statistical evaluation of the Tu-M2-PK values showed a highly significant difference (p<0.001) between the three groups (Table I). This highly significant difference existed both between the CIN and the cervical carcinoma groups (p<0.001) and between the CIN and the relapse group (p<0.001). In addition, there was a significant difference between invasive carcinoma (FIGO I-IV) and relapse (p<0.001) (Figure 1).

As shown in Figure 1 patients with FIGO stage III and IV carcinoma had a higher plasma concentration of Tu-M2-PK than patients with FIGO stage I carcinoma, although the difference just fell short of satisfying the 5% significance level (p=0.055). The differences between FIGO I and FIGO II patients (p=0.246), as well as between FIGO II and FIGO III/IV patients (p=0.380) were not statistically significant.
The comparison of all FIGO I versus FIGO II versus FIGO III/IV versus relapse showed a highly significant difference ($p<0.001$), which was confirmed in the comparison of two sample groups, with the relapse group being compared with FIGO I ($p<0.001$), FIGO II ($p<0.001$) and FIGO III/IV ($p=0.014$). The Spearman rank correlation coefficient of $r_s=0.678$ ($p<0.001$) showed a strong association between the Tu-M2-PK concentration and the stage of the disease.

In our patient population, Tu-M2-PK showed a diagnostic sensitivity for cervical carcinoma of 65.3% and a specificity of 86.0% using the recommended cut-off of 15.0 U/ml. The sensitivity of the tumor marker in the different tumor stages rose from 40.9% in FIGO stage I to 53.8% and 68.8% in stage II and III/IV, respectively, to 91.7% in patients with a relapse of cervical cancer.

The ROC curve shown in Figure 2 (AUC 0.842, 95% confidence interval: 0.774-0.911; $p<0.001$) gives an overview of the sensitivity and specificity of the test. In order to achieve a test specificity of at least 90.0% with the current patient population, a cut-off value of 16.0 U/ml would have to be chosen. In this case, the sensitivity would be 64.0%. The odds ratio of becoming sick was 1.170, with a significance of $p<0.001$.

With respect to lymph node metastasis the correlation coefficient of $r_s=0.524$ ($p<0.001$) shows that there was a positive correlation. The mean Tu-M2-PK concentration in the group with affected lymph nodes (N1) was significantly higher than in the node-negative group ($p=0.028$) (Table I). When distant metastasis was taken into account, a highly significant difference in Tu-M2-PK values was obtained ($p<0.001$). Patients with distant metastasis (M1) had distinctly higher values than patients without (M0). The correlation analysis showed that the presence of metastasized carcinomas were significantly associated with heightened Tu-M2-PK values ($r_s=0.486$ with $p<0.001$) (Table I). Divided into three different groups, without metastasis (N0/M0), with positive lymph nodes without distant metastasis (N1/M0), and with distant metastasis (M1), once again, a highly significant difference in Tu-M2-PK concentration between the groups was shown (with $p<0.001$), with the highest values being found in the group with distant metastasis (Table I). Increasing tumor dissemination was correlated not only with an increase in the tumor marker, but also with an increase in the sensitivity of the marker: from 47.8% in the N0/M0 group and 55.6% in the N1/M0 group, the sensitivity of Tu-M2-PK rose to 100% in the M1 group. With respect to lymph gland invasion and angioinvasion ($p=0.653$ and $p=0.931$) there was no significant correlation with the Tu-M2-PK concentration.

Of the 75 patients (51 patients with primary cervical cancer and 24 patients with relapse of cervical cancer), 58 had squamous cell carcinoma, 8 had an adenocarcinoma and 5 had an adenosquamous carcinoma (2: not classified/ 2: other carcinoma). Analysis showed that patients with squamous cell carcinoma had significantly higher concentrations of Tu-M2-PK than patients with adenocarcinoma ($p=0.035$). There was no significant correlation between the Tu-M2-PK concentration and the tumor grading (G 1-3) of cervical carcinoma ($p=0.349$).
A possible correlation between HPV status and Tu-M2-PK concentration was investigated in the precancerous group. In the general analysis of CIN I plus II with CIN III, the p-value (0.626), showed no significant difference between the stages. There was also no significant difference in Tu-M2-PK between the 18 patients with a HPV-negative finding and the 20 patients with a HPV high-risk positive finding (p=0.207). With respect to the question of whether the precancerous grade played a role in this context, CIN stages I and II were compared with CIN stage III, but there was no significant difference in the dysplasia grades, neither in the HPV-negative group (p=0.556) nor in the HPV-positive group (p=0.924) (Table I).

As for the question of whether age played a role in the Tu-M2-PK concentration, a correlation coefficient of r_s=0.118 indicated that there was no such connection. There was also no significant correlation between concentration of the marker and smoking behaviour of the patients (p=0.561), non-smokers: n=47; mean: 23.3 U/ml, standard deviation 24.3 U/ml; smokers: n=45; mean: 27.4 U/ml, standard deviation: 41.8 U/ml, range: <5.0-205.0 U/ml).

In 31 of the women with histologically confirmed cervical carcinoma, the SCC-AG concentration was determined retrospectively. The comparison of the SCC-AG concentration in the individual tumor stages showed a significant difference between them (p=0.015), as well as a strong correlation with the tumor stage (r_s=0.585; p<0.001). Lymph node-positive patients showed significantly higher SCC-AG concentrations than lymph node-negative patients (17.6 μg/l versus 0.9 μg/l; p=0.007; r_s=0.365 with p=0.044). Patients with metastasis also had significantly higher SCC-AG concentrations (21.8 μg/l versus 3.6 μg/l; p=0.005; r_s=0.409 with p=0.022). After the patient population was divided up into the groups N0/M0, N1/M0 and N1/M1, there were significant differences, with increasing concentration associated with progressive tumor dissemination (p=0.006).

The Tu-M2-PK and SCC-AG concentrations correlated significantly (r_s=0.587, p=0.001). In FIGO stage I, 3/6 of the cases had an Tu-M2-PK level above the cut-off value, whereas only 2/6 had a elevated SCC-AG value (Table II). In stage II, both markers were increased in 4/6 of the cases, and in stage 3 Tu-M2-PK was raised in 3/5 of the cases, SCC-AG in 4/5. For relapsed patients, 14/14 showed both heightened Tu-M2-PK and SCC-AG values (Table II).

Discussion

In various tumor marker guidelines, only SCC-AG and CEA are recommended for cervical carcinoma for therapy surveillance and early detection of a relapse. However, their clinical benefit has been questioned and has not yet been answered by a prospective clinical trial (33).

Table II. Sensitivity of the tumor markers Tu-M2-PK and SCC-AG for the different stages of the disease (n=31).

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Tu-M2-PK&gt;15 U/ml (x/n)</th>
<th>SCC-AG&gt;1.5 μg/l (x/n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO I</td>
<td>3/6</td>
<td>2/6</td>
</tr>
<tr>
<td>FIGO II</td>
<td>4/6</td>
<td>4/6</td>
</tr>
<tr>
<td>FIGO III</td>
<td>3/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Relapse</td>
<td>14/14</td>
<td>14/14</td>
</tr>
</tbody>
</table>

x/n: Number of patients (x) for the individual stages of the disease with a value above the defined cut-off value of 15.0 U/ml for Tu-M2-PK or 1.5 μg/l for SCC-AG, related to all the patients in this group (n). Only patients for whom both Tu-M2-PK and SCC-AG values were available are included in this analysis (n=31).

In the present study, we showed a high sensitivity of Tu-M2-PK with respect to invasive cervical carcinoma. We observed a significantly increased plasma concentration (p<0.001) in patients with a carcinoma as compared with patients with a pre-malignant lesion (CIN). A significant increase (p<0.001) was shown between the FIGO stages I, II, III/IV and the relapse status.

In the sole study so far of Tu-M2-PK in cervical carcinoma, by Kaura et al., a statistically significant difference in value was reported between healthy patients or patients with benign lesions and patients with carcinoma (32). In their patient population, Kaura et al. chose a cut-off value of 17 U/ml for distinguishing malignant lesions from non-malignant conditions. Under these conditions, the sensitivity was 82%, but the specificity was only 60% (32). In our patient population, with a cut-off value of 15 U/ml, there was a diagnostic sensitivity of 65.3% and a specificity of 86%. In order to be able to achieve a test specificity of 90% with our population, a cut-off value of 16 U/ml would have had to be chosen. In this case, the sensitivity would have been 64%. With a specificity of 90%, the best sensitivities to date, of up to 70%, have been achieved for gastrointestinal cancer (8, 9, 11, 12).

With increasing FIGO tumor stage, we observed an increasing concentration of Tu-M2-PK (correlation coefficient r_s=0.678). In the study, by Kaura et al., because the number of patients was too low, no comparison with respect to the tumor stages was carried out. Nonetheless, the authors spoke of at least a tendency for stage III patients to have higher values than stage II patients (32).

The sensitivity of Tu-M2-PK as a tumor marker increased from 40.9% for FIGO stage I to 91.7% for patients with a cervical carcinoma relapse. In the literature, an increase of the sensitivity of Tu-M2-PK as a tumor marker for progressive stages has also been shown for renal and lung carcinoma (21, 25).
With increasing metastasis of the cervical carcinoma, the sensitivity of Tu-M2-PK rose from 47.8% for the N0/M0 stage, to 55.6% for the N1/M0 stage and to 100% for the N1/M1 stage. For renal cell carcinoma, Roigas et al. observed a difference in the sensitivity of about 40% between M0 patients and M1 patients (20). On the other hand, for patients with gastrointestinal tumors, the difference in the sensitivity between M0 and M1 of 3-8% was very low (11).

The Tu-M2-PK values were clearly raised in the patients with metastasized cervical carcinoma (M1) in comparison with patients with non-metastasized carcinoma (M0) \((p<0.001)\). In the literature, this has also been shown for renal cell carcinoma (22), mamma carcinoma (36), and gastrointestinal tumors (9, 11, 12).

With respect to lymph node status (N), Tu-M2-PK concentration in the node-positive group (N1) was significantly higher than in the node-negative group (N0) \((p=0.028)\). To date, a significant correlation between high Tu-M2-PK values and positive lymph node status had only been shown for mamma carcinoma (31). Although the standard for statistical significance was not met with \(p=0.052\), node-positive (N1/M0) patients had higher concentrations of Tu-M2-PK than node-negative (N0/M0) patients. On the other hand, there was a clearly significant correlation \((p<0.001)\) between these two groups and the group of patients with distant metastasis (N1/M1). A comparable correlation between Tu-M2-PK and these three stages of tumor dissemination has been determined by Cerwenka et al. and Ventrucci et al. for pancreatic carcinoma (13, 15). In contrast, cervical lymph vessel and blood vessel \((L/V)\) invasion seems to have no significant correlation with the Tu-M2-PK concentration in patients with cervical carcinoma.

Analysis with respect to histopathological typing of the cervical tumor showed that only squamous cell carcinoma as compared to adenocarcinoma on average correlated with a higher Tu-M2-PK concentration \((p=0.035)\). In contrast, for lung carcinoma, Schneider et al. observed a higher plasma concentration for adenocarcinoma than for squamous cell carcinoma (25). For kidney carcinoma (19, 21) and testicular carcinoma (34), no significant difference between the histological subtypes was observed. In our patient population, other histological subtypes did not show a significant correlation.

There was no significant difference of the concentration of Tu-M2-PK between the stages of differentiation (G1-G3) in cervical carcinoma \((p=0.349)\). Roigas et al. observed significantly higher concentrations in G3 neoplasms than in G2 neoplasms (19). Furthermore, patients with an invasive ovarian carcinoma showed significantly higher Tu-M2-PK concentrations in late tumor stages with minimally differentiated tumors in comparison with early tumor stages and well-differentiated tumors (32).

The E7 oncoprotein of the human HPV 'high-risk' type 16 binds directly to M2-PK resulting in a transformation from the tetrameric to the dimeric form and the stabilization of dimeric M2-PK. It seems reasonable to suspect that this would result in a higher Tu-M2-PK concentration in HPV ‘high-risk’-positive patients than in HPV-negative patients. However, in the subgroup of our patient population with an intra-epithelial cervical neoplasm (CIN), no significant difference was observed in HPV ‘high-risk’-positive patients \((p=0.207)\). Subgroup analyses of the degree of precancerous alteration also showed no significant differences in concentration of Tu-M2-PK between the groups CIN I/II and CIN III, neither in HPV-positive nor in HPV-negative patients.

A continuous increase in the SCC-AG concentration, dependent on the tumor stage, was shown in women with invasive cervical carcinoma \((n=31)\). In stage I 33%, in stage III 80% and in the relapse stage 100% of the patients showed increased serum concentrations above the cut-off value. A total of 44% of the non-metastasis patients, 83.3% of the lymph node-positive patients and 100% of the relapse patients showed increased values.

Tu-M2-PK showed a higher correlation \((r_s=0.678)\) with the disease stage than did SCC-AG \((r_s=0.585)\). In lower tumor stages, Tu-M2-PK as a marker showed a greater increase, and therefore a greater sensitivity, than SCC-AG: in stage I, 50% of the cases had increased Tu-M2-PK levels, whereas only 33% had an increased level of SCC-AG. On the other hand, with regard to all tumor stages, SCC-AG proved to be more sensitive than Tu-M2-PK (sensitivity: 77.4% as opposed to 64.3%). The two markers correlate significantly with each other \((r_s=0.587; p<0.001)\).

In the diagnosis of lymph node metastasis SCC-AG had a sensitivity of 83.3%, whereas Tu-M2-PK only had a sensitivity of 55.5%. Nonetheless, Tu-M2-PK had a higher correlation with the presence of both lymph node and distant metastasis \((N: r_s=0.524\) and M: \(r_s=0.486)\) versus SCC \((N: r_s=0.365\) versus M: \(r_s=0.409\)).

Overall, it can be concluded that one third of the women with invasive cervical carcinoma had only one of the two markers in the pathological range. Combining both markers would seem to promise an increase in sensitivity.

**Conclusion**

The concentration of Tu-M2-PK in plasma correlates significantly with tumor progression and higher disease stage. There are clearly higher plasma levels in patients with malignant lesions as compared with patients with pre-malignant lesions. Furthermore, the highest concentration is found in patients with a tumor relapse. There is no significant correlation between the Tu-M2-PK concentration and lymph vessel and blood vessel invasion, the degree of differentiation, or the HPV status.
Tu-M2-PK qualifies as a new additional tumor marker for cervical carcinoma, especially with regard to the question of dissemination of the tumor. The sensitivity of the determination increases with the degree of dissemination, here reaching 100% in the case of relapse. This means that Tu-M2-PK should be suitable for therapy surveillance in advanced tumor stages. Further clinical studies of Tu-M2-PK concentration during treatment of large patient populations are desirable to provide support for these conclusions. The significantly increased plasma concentration in lymph node-positive as compared with lymph node-negative cases, and for increasing tumor stage, offers the hope that it will be possible to make pre-therapeutic decisions more accurately with the help of this marker. For example, the evaluation of operability given current imaging procedures, which have a low sensitivity, could be supported by determination of the plasma Tu-M2-PK concentration.

Further clinical studies are truly warranted.

References


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