Abstract. The aim of this study was to find a possible clinical use of the tail-interacting protein of 47 kDa (TIP47) and further document its expression in smear cytology, different cervical dysplasias, invasive cervical cancer and metastasis. Patients and Methods: A new polyclonal anti-TIP47 antibody was developed and used on smears and histological cervix sections of sixty women with different cytological pathologies. Serum TIP47 level of patients with cervical intraepithelial neoplasia (CIN) or carcinoma in stage IIb, IIIa, and IIIb was monitored during treatment. Results: TIP47 was expressed weakly in the dysplasias, stronger in invasive tumors and in lymph node metastasis. In patients with cervical carcinoma, the serum TIP47 level was found to be elevated; it decreased after therapy and elevated again in relapse. Conclusion: According to our results, TIP47 could be a good clinical marker for the early detection in blood of the recurrence of cervical carcinoma.

An increasing number of articles reported studies of tumor suppressor genes, oncogenes, cytogenetic abnormality and human papilloma virus typing, and their role in cervical cancer. However, sufficient evidence of their use as tumor markers in clinical practice has not yet been gathered. Thus, it has become particularly urgent that thorough research be carried out on the role of oncogenes in carcinogenesis with the aim of diagnosing cervical cancer at the earliest stage possible.

Evaluation of the p-STAT3 (transcription factor) expression in cervical intraepithelial neoplasias (CIN) was found to be significantly correlated with CIN lesion grade and cell proliferation (1). Correlation was demonstrated between the expression of the well-studied protein p16INK4a, a member of the INK4 family of cell cycle regulatory proteins, and the tumor suppressor protein pRb in cervical neoplasias, indicating that p16INK4a might be a specific marker for premalignant and malignant lesions of the squamous and endocervical mucosa (2). A further study showed significant association between cervical lesion grade and p16INK4a expression (3). It was also demonstrated that p16INK4a might be a biomarker for predicting the recurrence of CIN and its progression to cervical cancer (4).

Lipid droplets are specialized organelles containing lipids and proteins on their surface that participate in lipid metabolism and intracellular trafficking. In mammals, the best-characterized surface proteins belong to the PAT family, so-named for their core members: perilipin, adipose differentiation-related protein (ADRP) and tail-interacting protein of 47 kDa (TIP47) (5-9), which was originally described as soluble placental tissue protein 17b (10). During pregnancy, TIP47 serum levels increase; after birth they drop (10, 11). Although some TIP47 is found on lipid droplets, TIP47 is known to be required for the delivery of mannose 6-phosphate receptors from late endosomes to the Golgi, both \textit{in vitro} and in living cells (6). The protein binds the cytoplasmic domains of the cation-dependent and cation-independent receptors (12, 13), and is recruited to late endosomes by binding to Rab9 GTPase (14, 15). The loss of TIP47 destabilizes Rab9 (16) which is also required for proper receptor transport. Lysosomal enzymes, such as prostate-specific antigen (PSA), are often markers of cancer. We might assume that the surface location of TIP47 is a property that classifies it as a putative marker of cancer.

In the past four years, the oncological significance and specific overexpression of TIP47 in human uterine squamous cervical carcinoma tissues and HeLa (squamous cervical cancer) cells were established. Serum TIP47 levels were found to be elevated in cervical carcinoma patients, and declined after radical surgery. By immunohistochemistry, normal cervical epithelia were negative for TIP47 (PP17b),
while in low-grade dysplasias moderate positivity, and in high-grade dysplasias strong positivity were found. In invasive squamous cervical carcinomas, the cytoplasm of basal-type tumor cells were negative, while squamous-type dysplastic cells were strongly positive (10, 11, 17-19).

The purpose of this study was to examine the possible application of our newly developed anti-TIP47 antibody for cervical smear screening by comparing it to the standard cervical smear test, and to examine the serum TIP47 level and its expression in different cervical dysplasias, invasive cervical cancer and metastases to establish a possible clinical use as a new biomarker.

Materials and Methods

Cell culture. NIH3T3, HeLa, HepG2, Panc-1 and WRL-68 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 units/ml penicillin-streptomycin mixture and incubated in 5% CO₂-95% air at 37°C. Cells were harvested and low-speed centrifuged, then the pellet was dispersed by vortexing in lysis buffer [50 mM Tris pH 7.4, 1 mM phenylmethylsulfonyl-fluoride (PMSF)] for 10 min at 4°C.

Construction of bacterial TIP47 expression plasmid. The full length TIP47 cDNA clone was obtained from RZPD (Berlin, Germany). From the target sequence, containing the full length TIP47 cDNA, the sequence was PCR amplified with TIP47-specific primers (forward: 5' ATG TCT GCC GAC GGG GCA G and reverse: 5' AGC TGC ATT ATA GAG ACG G). The resultant PCR products were cloned into pGEX-4T-1 expression vector (Invitrogen, Carlsbad, CA, USA).

Expression and purification of TIP47. The TIP47/pGEX-4T-1 expression vector was transfected into Escherichia coli DH5α competent host strain. After isolation of the plasmid, it was transfected into E. coli BL21 host strain. Bacteria were induced with β-D-1-thiogalactopyranoside (IPTG), and the expressed protein was subsequently purified with a glutathione sepharose 4B column in the presence of glutathione, after which thrombin (Sigma, St. Louis, MO, USA) cleavage was carried out. The primary structure and purity of TIP47-recombinant protein was verified by sequence analysis.
Figure 2. Expression pattern of TIP47 in HeLa cells (A), control cervix (B), CIN 1 (C), CIN 2 (D), CIN 3 (E), in invasive carcinoma (F), and in lymph node metastasis (G) as detected by immunocytochemical and immunohistochemical staining with anti-TIP47 antibody. Strong cytoplasmic staining could be seen in the HeLa cells on the cytospin preparation (A). With increasing CIN severity (C-E), cytoplasmic TIP47 immunostaining is detected in dysplastic cells from the basal layer into the superficial layer. In invasive carcinoma (F), areas with squamous differentiation and keratinization show stronger staining, while in the metastasis (G), a moderately punctuated pattern can be seen. The control section (B) showing healthy cervix histology displays a very weak staining of TIP47 (original magnification ×400).
Preparation of polyclonal antibodies against TIP47. Two rabbits were immunized subcutaneously at multiple sites with 100 μg of recombinant TIP47/GST fusion protein (with GST located at the N-terminal of the fusion protein) in Freund’s complete adjuvant (Sigma). Four subsequent booster injections at 4-week intervals were given with 50 μg of protein in Freund’s incomplete adjuvant (Sigma). Blood was collected 14 days after boosting, and the antisera were stored at −20°C. The IgG fraction was isolated from the sera by protein G-Sepharose chromatography and affinity purified using recombinant TIP47 recombinant protein bound to a CNBr activated Sepharose 4B column and affinity chromatography as described elsewhere (20).

Patients, cytology and blood samples. This study received the approval of the Ethics Committee of the Medical University of Pécs. Human tissue and sera from patients were provided by the Departments of Pathology and Obstetrics and Gynecology, Baranya County Teaching Hospital of the Medical University of Pécs. Two cytological smears were taken from each patient. The first slide from each patient was pathological examined according to the Bethesda System (TBS 2001) and fifteen samples were selected randomly from each Bethesda group (normal, atypical squamous cells of undetermined origin (ASCUS), low-grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL)). The second slide was stained with anti-TIP47 antibodies and counterstained by hematoxylin. Smears with LSIL or HSIL cytological alterations were repeated and when necessary conization was carried out, providing tissue samples for immunohistochemical diagnosis.

Blood was collected from patients with CIN (10 CIN1, 10 CIN2, 10 CIN3) diagnosis and 9 FIGO stage Ib, 10 IIa, 8 IIb, and 8 IV cervix carcinoma patients, before, and 6 days, 6 weeks, and half year after the appropriate treatment. Patients were treated with external beam irradiation in combination with cisplatin. For controls, sera were collected from 5 healthy adult men, 5 women and 2 sera from each of the following cancer type: colorectal, pancreatic, brain, ovarian, breast, lung, liver, and kidney. Sera were separated by ultracentrifugation and equal amounts were diluted in standard Laemmli sample solution.

SDS-PAGE / Western blot. Ten μg of proteins from the cell lines, tissue extracts and equal amount of sera were subjected to 12% SDS-PAGE (w/v). In addition a dilution series from the expressed TIP47 protein was also processed likewise. Immunoblots were carried out with anti-TIP47 antibody and horseradish peroxidase-labeled secondary IgG as described elsewhere (21). Protein bands were revealed by an ECL chemiluminescence system followed by quantitative densitometry using ImageJ for Windows.

Cervical smear. Cervical smears were prepared in the conventional manner and classified according to the Bethesda System.

Immunolocalization of TIP47 in Hela cells, normal cervix, cervical carcinomas and metastasis. HeLa cells were spread on glass slides with cytospin (Shandon, USA). After a 20-min fixation in 4% ice-cold paraformaldehyde (PFA) the endogenous peroxidase was inhibited by phenylhydrazine hydrochloride (Sigma; 1 mg/ml in phosphate-buffered saline, PBS). Nonspecific binding was blocked with 5% bovine serum albumin (BSA) saturation for 20 min. Tissue microarray (TMA) blocks were prepared from previously histologically examined routine formalin-fixed, paraffin-embedded cervical tissue samples including normal or dysplastic cervix or invasive squamous cervical carcinoma cases using the manual TMA builder according to instructions of the manufacturer (Histopathology, Ltd., Pécs, Hungary). Four-μm TMA sections were cut, mounted on slides, dewaxed in xylene, rinsed in ethanol, and endogenous peroxidase activity was blocked in 0.5% hydrogen peroxide in methanol (30 min). Sections were rinsed in 70% ethanol, tap water, distilled water, and Tris-buffered saline, pH 7.6 (TBS). The sections were then incubated in 20% normal rabbit serum (NRS) in TBS to block nonspecific binding and then incubated at room temperature. HeLa cytosin preparation and tissue sections were incubated with anti-TIP47 antibody diluted to 1:500. Immunostaining was carried out according to the streptavidin-biotin-peroxidase technique, with hydrogen peroxide/3-amino-9-ethylcarbazole development using Ventana DAB Universal Kit (Ventana-Bio Tek Solutions, Tucson, AZ, USA). Visual evaluation of hematoxylin-counterstained slides was performed using an Olympus BX50 light microscope with incorporated photography system (Olympus Optical Co., Hamburg, Germany).

Statistical evaluation. Values in the figures, tables and text are expressed as mean ± SEM of n observations. Statistical analysis was performed by analysis of variance followed by Student’s t-test. Statistical significance was set at p<0.05.

Table I. A) Immunocytological staining of TIP47 in cervical cytological samples.

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<th>Immunocytological reaction for TIP47 n (%)</th>
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<tr>
<td></td>
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<td>Normal</td>
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B) TIP47 staining on histological sections compared with repeated and confirmed preoperative cytology.

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<th>Immunohistology n (%)</th>
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<tr>
<td>Cytology</td>
<td>Non-dysplastic</td>
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<td>ASCUS</td>
<td>(0.00)</td>
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<tr>
<td>HSIL **</td>
<td>(6.67)</td>
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ASCUS: Atypical squamous cells of undetermined origin, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion. *Conization results of LSIL cases (mostly negative) were significantly matched to cytology results stained with anti-TIP47 antibodies (p<0.01); **Conization results of HSIL cases matched to cytology results stained with anti-TIP47 antibodies were not significant (p>0.05).
Results

The new polyclonal anti-TIP47 antibody. The new polyclonal antibodies were tested by Western blots and by immunocytochemical staining of HeLa cells on cytospin preparation. By Western blot, a strong band could be detected at 48 kDa with the cleaved, recombinant TIP47 protein and HeLa cell extract, in which high TIP47 expression was seen before (3). Very weak staining was seen in Panc-1, WRL-68 and Hep-G2 cell line extracts, and no sign of reaction in the NIH3T3 cell line (Figure 1). With immunocytochemistry, strong cytoplasmic staining was detected in HeLa cells on cytospin preparation (Figure 2A). In short, our affinity purified anti-TIP47 antibody was found to be highly specific and worked well by Western blot and immunocytochemistry.

Immunolocalization of TIP47 in cervical dysplasias, squamous cervical carcinoma and in metastatic tissue sections. Immunohistochemical staining showed that TIP47 was predominantly localized in the cytoplasm of the epithelial cells. In cases of CIN I (Figure 2C), very weak cytoplasmic positivity was detected. In CIN II (Figure 2D), the basal layer of the epithelium was moderately stained and

Figure 3. Serum levels of TIP47 in different cervical pathologies as detected by Western blot. Human sera were obtained from patients with different stages of dysplastic or tumorous cervical squamous cell lesions. A, The Western blot samples are representative of each stage examined. Sera were obtained from patients in the following order: healthy control; CIN1, CIN2, CIN3; invasive carcinoma before treatment, invasive carcinoma 6 days (6d), 6 weeks (6w), and 6 months (6m) after treatment; lymph node metastasis (Met). B, Analysis of serum TIP47 levels of 65 patients by quantifying Western blot results. Bars show the corresponding percentage of elevation in TIP47 serum levels as compared to healthy control values. Values are expressed as means ± S.E.M. of all serum samples measured three times, and quantified from Western blot images by ImageJ software. Significantly different from the control at *p<0.05, **p<0.01.
also granulated; in the superficial part of the epithelium weaker staining could be seen. In high-grade dysplasia CIN III (Figure 2E), all layers of the whole epithelium showed more intense granular positivity. In invasive cervical carcinomas, heterogeneous cellular distribution of granular cytoplasmic immunostaining was detected (Figure 2F): the small basal-type tumor cells were mostly moderately positive and in the areas with squamous differentiation and keratinization stronger staining was seen. In a cervical cancer metastasis (Figure 2G), TIP47 was also expressed moderately and a punctuated pattern was also present.

The possible application of TIP47 for staining in cervical cytology. Our aim was to compare the immunological staining with anti-TIP47 antibodies to the conventional smear test by the Bethesda System on cervical cytology. When staining normal or ASCUS cytological samples, we could not detect TIP47 in most cases (10/15 and 9/15 respectively), but in a few instances we found mild or moderate staining. On the other hand, TIP47 staining of LSIL and HSIL cytological samples showed doubtful results. We could detect samples with no TIP47 staining in 26.6% of the samples in both cases, but the number of mildly or moderately stained samples were also high (6/15 or 4/15; and 3/15 or 5/15 respectively) and we could even detect intensely positive cells (1/15 and 3/15, respectively) (Table IA). Table IB shows the histological results of the conizations after the repeated and confirmed Bethesda cytology. By comparing TIP47 immunocytochemical staining to conventional smear tests, we detected significant correlations only in cytologically negative cases. We also tried to predict the outcome of the histological results from immunocytochemistry, but results were very doubtful and significant correlation could not be shown. We conclude that immunostaining with anti-TIP47 antibody provides no further information when compared to conventional smear tests.

TIP47 as a new serum marker for detecting cervical cancer. Analyzing the TIP47 serum levels of patients with dysplasias or in situ cervical carcinomas, we did not find any considerable elevation compared to the serum levels of healthy adult females or males. The TIP47 serum level was also not elevated in colorectal, pancreatic, brain, ovarian, breast, lung, liver or kidney cancer (data not shown). Comparing the normal serum levels with the dilution series from the expressed TIP47 protein by Western blot, the TIP47 serum level was estimated to be about 500 ng/ml (data not shown). Nevertheless, when cancer cells invaded the basal membrane of the epithelium, and the tumor became invasive, a significant elevation of TIP47 could be detected in the serum. Six days after the appropriate treatment, TIP47 values dropped and they decreased to approximately normal levels at 6 weeks after treatment and were found to be stable even after six months following the end of the treatment. During the follow-up period, eight patients, five in Stage IIB and three in Stage IIIA, who developed local recurrence as confirmed by magnetic resonance imaging (MRI) had elevated levels of TIP47 in sera as detected by Western-blot (Figure 3A, B).

Discussion

A number of proteins have been implicated in the development and progression of cervical cancer (1, 3, 18, 22-26), but little is known of the processes taking part in the transformation of cervical dysplasia into invasive cancer. The search is still on to find more specific prognostic markers of cervical premalignant lesions and invasive squamous cervical cancer, such as expression of certain cancer-related proteins. The study of these proteins may bring us closer to understanding the process of oncogenesis and thus to predicting the transformation of dysplastic lesions and forecasting their clinical outcome.

It has been reported that TIP47 (PP17b) is overexpressed in cervical dysplasias and in invasive carcinoma and that it is secreted into the circulation in increased amounts in patients with invasive cervical carcinoma and during pregnancy (10, 18). TIP47 belongs to the lipid droplet-associated PAT protein family (perilipin, ADFP, and TIP47), and considerable data have accumulated in connection with its function, thus supporting the hypothesis that TIP47 plays an important role in lipid metabolism (27-30). The reason for its specific and high expression in cervical malignancies and its possible role in the multiple steps of cervical cancerogenesis are, however, still unclear.

In this study we sought to determine a clinical importance of this unique protein by exploiting its unique properties. We were curious whether TIP47 could be used in the early detection of cervical carcinoma. Smears of sixty women with different cytological pathologies classified by the Bethesda System were immunocytologically examined using in-house created anti-TIP47 polyclonal rabbit antibody. The antibody was previously tested on different cell lines to demonstrate its specificity (Figure 1). The result of this comparative experiment gave us no further information about the possible outcome of the disease; in fact, a very doubtful result was obtained (Table I). In search for a clinical use of this protein, we performed immunohistochemical staining of different dysplastic, invasive and metastatic tumors with our anti-TIP47 antibody. Our results were similar to those previously described (18), in CIN I, CIN II and CIN III moderate staining of the dysplastic cells could be seen, but punctuated anti-TIP47 staining could be detected in invasive tumor and also in a lymph node metastasis (Figure 2). The sera of patients with dysplasias and invasive cancer were also
examined before and after an appropriate radiochemotherapy treatment. Western blots were carried out to estimate the level of TIP47 in the sera. TIP47 was not elevated in the sera of patients with precancerous lesions, but showed significant elevations in patients with untreated invasive or metastatic disease. At the completion of therapy, the levels of TIP47 started to decline and reached normal levels at the 6 week examination. In patients with no recurrence, the TIP47 level dropped to a normal level. However, in patients with recurrence, confirmed with MRI examination, the level of TIP47 increased.

Several previous studies have had similar findings. Mathur and colleagues showed that the serum IGF-II level increased in cervical dysplasias and carcinomas, and after the therapy it declined, thus they recommended it as a reliable marker for early diagnosis and for monitoring the efficacy of the therapy, while IGF-BP3 levels could be reliably used to predict prognosis (31). These data were confirmed, and moreover it was also demonstrated that measuring the serum vascular endothelial growth factor C (VEGF-C) level could provide an early noninvasive and specific diagnosis of potential metastasis in women with cervical cancer (32-34).

Others showed that both VEGF and VEGF-C concentrations increased significantly in patients with squamous cell carcinoma. The pretherapeutic serum levels of VEGF and VEGF-C correlated significantly with the stage and the tumor size, but not with lymph node metastasis. The pretherapeutic serum level of VEGF-C also correlated significantly with disease recurrence or persistence after treatment. Both serum VEGF and VEGF-C levels decreased significantly after treatment (35). It was also demonstrated that the serum level of the squamous cell carcinoma (SCC) tumor marker antigen correlated with the prognosis of the operable SCC of the cervix (36).

According to our results, we conclude that TIP47 is expressed in squamous cervical dysplasias and also in invasive tumors and in their metastases. It is secreted into the serum and can be monitored using immunological methods. The rationale of this study lies in the detection of serum TIP47 level in patients with invasive carcinoma, since the progression of the tumor to invasive or metastatic carcinoma will result in an elevated serum TIP47 level. Since the TIP47 serum level drops after treatment, we hypothesize it to be a good clinical marker for the early detection of the recurrence using blood, hence abolishing the need for the costly MRI examination. Since our examinations are preliminary, there is a need for a clinical study with greater number of patients.

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