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The novel serine protease tumor-associated differentially expressed gene-14 (*KLK8/Neuropilin/Ovasin*) is highly overexpressed in cervical cancer

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KEY WORDS

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Objective: Serine proteases are redundant enzymes implicated in the extracellular modulation required for tumor growth and invasion. Tumor-associated differentially expressed gene-14 (TADG-14) is a novel transmembrane serine protease recently reported by our group to be highly overexpressed in ovarian carcinomas. The goal of this study was to investigate the frequency of expression of the TADG-14 gene in human cervical tumors.

Study design: TADG-14 expression was evaluated in 19 cervical cancer cell lines (11 primary and 8 established cell lines) as well as in 8 normal cervical keratinocyte cultures by reverse transcriptase polymerase chain reaction. In addition, to validate gene expression data at the protein level, TADG-14 expression was evaluated by immunohistochemistry on paraffin-embedded tissue from which all 11 primary tumor cell lines were established.

Results: TADG-14 was found to be highly expressed in 82% (9/11) primary cervical cancer cell lines and in 87% (7/8) established cervical cancer cell lines by reverse transcriptase-polymerase chain reaction. Expression of TADG-14 by primary squamous cervical tumors was 100% (6/6), whereas 60% (3/5) of primary adenocarcinomas expressed TADG-14. In contrast, none of the normal cervical keratinocyte control cultures (n = 4) or flash frozen normal cervical biopsy specimens (n = 4) expressed TADG-14. Immunohistochemistry staining of paraffin-embedded cervical cancer specimens confirmed TADG-14 expression in tumor cells and its absence on normal cervical epithelial cells.

Conclusion: Cervical cancer expressed a high level of TADG-14, suggesting that this protease may play an important role in invasion and metastasis. Because TADG-14 appears only in abundance in tumor tissue and contains a secretion signal sequence, suggesting that TADG-14 is secreted, it

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may prove to be a useful diagnostic tool for the early detection of recurrent/persistent cervical cancer after standard treatment or as a novel molecular target for cervical cancer therapy.
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Tumor invasion and metastatic spread is a multistep process that begins with the shedding of malignant cells from the primary site, transporting the cells through blood vessels, and seeding at distant organs, resulting in tumor-associated metastasis.¹⁻³ In the last few years, strong clinical and experimental evidence has accumulated that malignant cells depend on a group of proteolytic enzymes synthesized and secreted by tumor cells to disrupt basement membranes, invade neighboring tissues, and metastasize.¹⁻³ Consistent with this view, several reports have demonstrated a positive correlation between cancer progression and expression of extracellular proteases such as matrix metalloproteinases (MMPs) and serine proteases (urokinase-type plasminogen activator [uPA]) in a variety of human tumors.¹⁻⁴ Furthermore, inhibitors of these proteolytic enzymes have been shown to interfere with the spread of cancer in animal models.⁵ These findings have prompted efforts to develop novel anticancer therapies directed against the proteolytic cascade initiated by MMPs and serine proteases and the various signaling pathways mediated by these enzymes.

Serine proteases are a family of protein-degrading enzymes that play crucial roles in numerous biologic processes, including activation of complement, blood coagulation, degradation of extracellular matrix component, and activation of growth and angiogenic factors.⁴ All serine proteases contain conserved histidine, aspartate, and serine residues that are necessary for enzymatic activity and are either secreted enzymes or sequestered in cytoplasmic storage organelles awaiting signal-regulated release.⁴ During the last few years, several novel transmembrane serine proteases have been cloned and reported in humans including hepsin,⁶ tumor-associated differentially expressed gene-12 (TADG-12),⁷ TADG-14 (*KLK8/Neuropilin/Ovasin*),⁸ TADG-15 (*Matriptase*),⁹ TADG-16 (*Testisin*),¹⁰ stratum corneum chymotryptic enzyme,¹¹ seprase,¹² and protease M.¹³ Several of these membrane-spanning proteases have been reported to have cytoplasmic N-terminal domains, suggesting possible functions in intracellular signal transduction.⁴ Importantly, in prostate cancer, the secreted nature and/or high level of expression of the serine protease prostate-specific antigen (PSA) allows for its detection at aberrant levels in patient serum, making PSA an important tumor marker for the early diagnosis of this disease.¹⁴ In breast cancer, the serine protease uPA has also proven to be a useful negative prognostic marker for disease progression.¹⁵

To assess the value of secreted proteases as markers for tumor detection and targets for therapeutic intervention, our group has recently developed a screening strategy using redundant primers to the conserved catalytic triad domain of the serine protease family. By using this approach to display serine protease transcripts found in abundance in gynecologic tumor tissues, with little or no expression in normal tissues, we recently reported the identification and cloning of several transmembrane serine proteases, including TADG-14, in ovarian cancer.^{7-9,13} In this study we have evaluated the extent and frequency of expression of the TADG-14 gene in several primary and established squamous and adenocarcinoma cervical cancer cell lines. We report, to our knowledge for the first time, expression of TADG-14 on the majority of cervical tumors evaluated. These findings may have important implications for the use of TADG-14 protease as a diagnostic tool or as a molecular target for cervical cancer therapy.

Material and methods

Primary and established cervical cancer cell lines

Nineteen cervical cancer cell lines (ie, 11 primary and 8 established) were evaluated for TADG-14 expression by reverse transcriptase-polymerase chain reaction (RT-PCR). Commercially available established cervical cancer cell lines (ie, CaSki, SiHa, MS-751, HT-3, C33a, Me180, HeLa, and C4-I) were purchased from the American Type Culture Collection (Manassas, Va). Tumors derived from primary specimens were staged according to the International Federation of Gynecology and Obstetrics (FIGO) operative staging system. Fresh tumor biopsy specimens from patients who had frankly invasive stage IB-IIA cervical cancer diagnosed were obtained at the time of surgery through the Gynecologic Oncology Division and the Pathology Department, University of Arkansas Medical Sciences (UAMS), under the approval of the Institutional Review Board. Radical abdominal hysterectomy and lymph node dissection for invasive cervical cancer were performed in all patients but one. Eight normal keratinocyte control samples (ie, four primary keratinocyte cultures and four flash frozen biopsy specimens) were obtained from cervical biopsies of hysterectomy specimens from women who had benign disease diagnosed and a previous report of a normal cytologic evaluation.

Cervical cancer and normal keratinocyte cell lines were established according to previously reported standard tissue culture techniques.¹⁶ We studied 11 primary cervical cancer and 4 primary keratinocyte cell lines, some of which had been cultured for 3 to 4 weeks and others over a much longer period. Purity of fresh tumor cultures was tested by morphology, immunohistochemistry staining, and/or flow cytometry with antibodies against cytokeratins. Only cell lines containing more than 99% tumor cells were evaluated. Because tissue digestion and prolonged in vitro cell culture may potentially alter antigen expression, four adjunctive normal cervical keratinocyte biopsy specimens were also evaluated for TADG-14 expression before enzymatic digestion and/or in vitro culture (ie, snap frozen in liquid nitrogen immediately after collection).

RNA isolation and complementary DNA synthesis

RNA isolation from different cell lines was performed using TRIzol Reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. To verify integrity, 4 µg of RNA from each sample was run in 1% agarose gel using 18S+28S ribosomal RNA (Sigma, St Louis, Mo) as positive control. First-strand complementary DNA (cDNA) was synthesized by using 5 µg of total RNA, 1× RT-PCR buffer, 5 mmol/L magnesium chloride, 1 mmol/L dNTPs, 2.5 µmol/L random hexamers, 1 U/µL Rnase inhibitor, and 2.5 U/µL of MuLV reverse transcriptase (Gene Amp RNA PCR kit; Applied Biosystems, Foster City, Calif) in a total volume of 20 µL. RNA extracted from CaOV3 serous papillary ovarian cancer cell line, previously reported to express TADG-14,⁸ was used as a positive control.

PCRs

The PCR reaction mixture consisted of cDNA, 0.4 µmol/L of sense and antisense primers, 2.5 mmol/L magnesium chloride, 0.2 mmol/L dNTPs, 0.025 U/µL Taq polymerase (Gene Amp RNA PCR kit; Applied Biosystems) with reaction buffer in a total volume of 50 µL. The target sequences were amplified in parallel with β-tubulin gene.

Thirty cycles of PCR for specific target were performed in a GeneAmp PCR System 2700 (Applied Biosystems). Each PCR cycle included 30 seconds of denaturation at 95°C, 30 seconds of annealing at 67°C, and 30 seconds of extension at 72°C. The sequences of the TADG-14-specific primers that produce the 230-bp product were as follows: 5'-ACAGTACGCCTGGGA-GACCA-3' forward and 5'-CTGAGACGGTGCAAT-TCTGG-3' reverse. The sequences of β-tubulin primers that produce the 454-bp product were as follows: 5'-CGCATCAACGTGTACTACAA-3' forward and 5'-TACGAGCTGGTGGACTGAGA-3' reverse.

Antibody production and immunohistochemistry

Polyclonal antibodies were generated by immunization of New Zealand White rabbits with one of three polylysine-linked multiple antigen peptides derived from the amino acid sequence of TADG-14 as previously reported.⁸ These sequences are KYTVRLGDHSLQ (T14-1), GHECQPHSQPWQ (T14-2), and LDWIK-KIIGSKG (T14-3). Formalin-fixed, paraffin-embedded tissue blocks from eight normal uterine cervixes (ie, controls) and 11 invasive cervical carcinomas of the uterus (6 squamous carcinomas and 5 adenocarcinomas), from which primary cell lines were established, were retrieved from the surgical pathology files of the UAMS and evaluated for TADG-14 expression. Study blocks were selected after histopathologic review. The most representative hematoxylin and eosin-stained block sections were used for each specimen. Immunohistochemical staining was performed with a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, Calif). Briefly, formalin-fixed and paraffin-embedded specimens were routinely deparaffinized and processed by using microwave heat treatment in 0.01 mol/L sodium citrate buffer (pH 6.0). The specimens were incubated in methanol with 0.3% hydrogen peroxide for 30 minutes at room temperature and then incubated with normal goat serum for 30 minutes. The samples were incubated with anti-TADG-14 peptide-derived polyclonal antibody for 1 hour at room temperature in a moisture chamber, followed by incubation with biotinylated antirabbit immunoglobulin G (IgG) for 30 minutes, and then incubated with ABC reagent (Vector Laboratories) for 30 minutes. The final products were visualized by using the AEC substrate system (DAKO, Carpinteria, Calif), and sections were counterstained with hematoxylin before mounting. Negative controls were performed by using normal serum instead of the primary antibody.

Statistical analysis

Data were analyzed with the two-tailed *t* test and the Student *t* test for paired data. In all tests, the difference was considered significant when *P* values were less than .05.

Results

TADG-14 expression in primary and established cervical cancer cell lines

To characterize the frequency of expression of the TADG-14 gene in cervical tumors, we used RT-PCR with cDNA derived from primary and established squamous and adenocarcinoma cervical cancer cell lines as a template. Cultures of normal cervical keratinocytes were used as controls. All primary tumors studied were

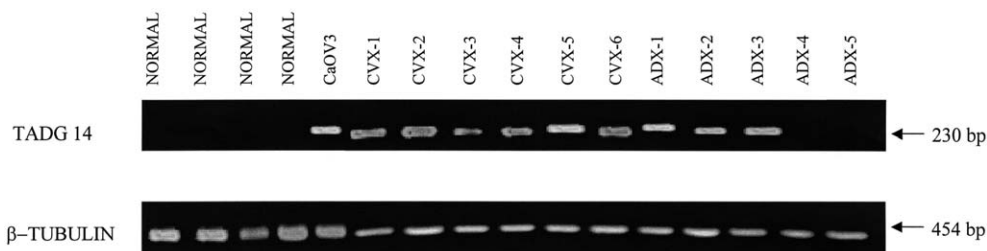


Figure 1 TADG-14 expression by RT-PCR on primary squamous cell carcinoma (CVX-1-6), primary adenocarcinoma (ADX1-5), and normal cervical keratinocyte primary cell lines. The reaction products were electrophoresed through a 2% agarose gel and stained with ethidium bromide. The 454-bp band represents the β -tubulin product, and the 230-bp band represents the TADG-14 product. TADG-14

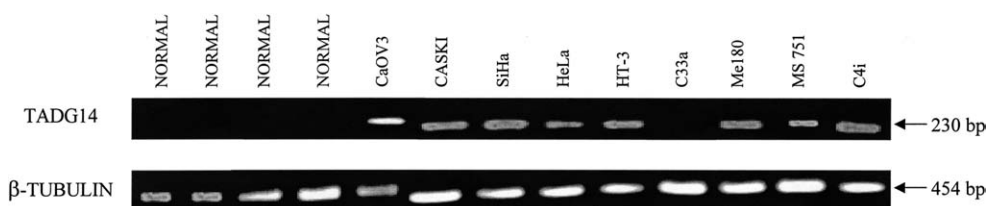


Figure 2 TADG-14 expression by RT-PCR on established cervical cancer cell lines and normal keratinocyte flash-frozen biopsy specimens. The reaction products were electrophoresed through a 2% agarose gel and stained with ethidium bromide. The 454-bp band

originated from patients diagnosed with stage IB (90%) or IIA (10%) cervical cancer (mean age \pm SD = 38 ± 8 years, range 26-50 years). Ten patients received surgery and 1 patient received radiation therapy as primary treatment. PCR primers that amplify a TADG-14-specific 230-bp product were synthesized and used in reactions. Primers that produce a specific 454-bp PCR product for β -tubulin were also used as internal controls. Of the 19 cervical carcinomas studied, 16 (84%) were found to express TADG-14 by RT-PCR. **Figure 1** shows an ethidium bromide-stained agarose gel with the separated RT-PCR products for normal cervical keratinocytes and primary cervical cancer cell lines, whereas **Figure 2** shows the RT-PCR results for the established cervical cancer cell lines. Both are representative of the typical results observed. Flash-frozen samples or cultures of normal cervical keratinocytes did not express TADG-14 (**Figures 1 and 2**). In contrast, TADG-14 was found to be expressed in 82% (9/11) of primary cervical cancer cell lines and in 87% (7/8) of established cervical cancer cell lines ($P < .001$). For individual histologic subtypes of primary tumors, TADG-14 expression by primary squamous cervical tumors was 100% (6/6), whereas for adenocarcinomas was 60% (3/5).

Immunohistochemistry staining

To determine whether TADG-14 expression detected by RT-PCR in primary cervical cancer cell lines is the result

of a selection of a subpopulation of cancer cells present in the original tumor or whether in vitro expansion conditions may have modified gene expression, we performed immunohistochemical analysis of TADG-14 protein expression on formalin-fixed paraffin-embedded tumor tissue from all primary tumors from which primary cell lines were derived. As shown in the **Table**, immunohistochemical staining supported the data obtained by RT-PCR. By using a TADG-14 peptide-directed antibody (T14-1), we observed no significant staining with normal cervical keratinocytes samples (**Figure 3, A**). In contrast, similar to ovarian tumor cells (**Figure 3, B**), intense staining was associated with cervical tumor cells of both squamous cell and adenocarcinoma histologic subtypes (**Figure 3, C and D**). For squamous carcinoma, the antigen appears to be associated with tumor cells in the form of fine cytoplasmic granules. These granular structures may be intermediates in the pathway that ultimately leads to the secretion of TADG-14. In adenocarcinoma samples (**Figure 3, D**), TADG-14 showed diffuse cytoplasmic staining.

Comment

The biologic aggressiveness of neoplastic cells lies in their ability to proliferate abnormally and to invade normal host tissues. Malignancies use proteases to provide a variety of services that assist in the process of tumor progression, including activation of growth and

Table TADG-14 expression in squamous and adenocarcinoma cervical cancers and normal keratinocytes by immunohistochemistry

Tissue type	TADG-14 expression
CVX 1	+
CVX 2	+
CVX 3	+
CVX 4	+
CVX 5	+
CVX 6	+
ADX 1	+
ADX 2	+
ADX 3	+
ADX 4	—
ADX 5	—
KRT 1	—
KRT 2	—
KRT 3	—
KRT 4	—

CVX, Squamous cervical cancer; ADX, adenocarcinomas; KRT, normal

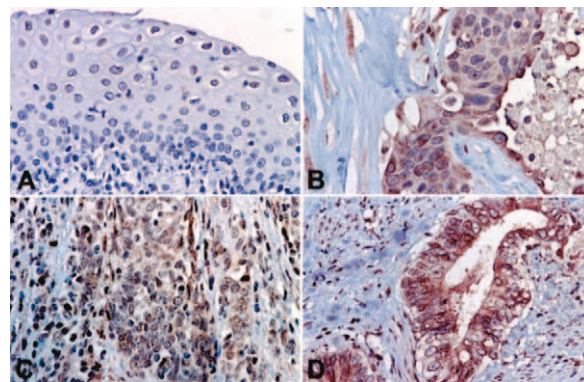


Figure 3 Immunohistochemical analysis of TADG-14 expression in cervical tumors. Representative immunoreactivity of TADG-14 in normal epithelial cells (A), ovarian serous papillary carcinomas (positive control) (B), cervical squamous cell carcinoma CVX-2 (C), and adenocarcinoma ADX-1 (D) (original magnification, $\times 300$). Although no staining is observed in normal cervical keratinocytes, in squamous cell carcinoma TADG-14 appears to be most highly expressed along the invasive front of the

angiogenic factors, and to provide the basis for invasion and metastasis. Consistent with this view, previous work has shown significant changes in matrix MMPs and their endogenous inhibitors during cervical tumor progression.^{17,18} In the process of studying proteolytic enzymes playing a key role in tumor metastatic processes, we have recently identified overexpression of TADG-14 (Table) as well as other serine proteases in a high percentage of ovarian cancers.^{7-9,13} Because of the paucity of information on serine protease expression in cervical carcinomas, in this work we have analyzed the expression of the novel serine protease TADG-14 in several short-term primary cultures as well as established cervical cancer cell lines. We chose this approach to evaluate differential TADG-14 gene expression in highly enriched populations of cervical tumor-derived epithelial cells. We report for the first time that TADG-14 is expressed in 84% (16/19) of the cervical carcinomas studied, whereas it is undetectable in normal cervical keratinocytes. These data therefore support the view of TADG-14 as a highly differentially expressed serine protease in cervical tumors when compared with normal control cells (ie, cervical keratinocytes). Furthermore, to validate gene expression data at the protein expression level, TADG-14 expression was evaluated by immunohistochemistry on paraffin-embedded tissue from which all 11 primary tumor cell lines were established. All primary uncultured tumor specimens, from which TADG-14 expression was detected by RT-PCR, express detectable TADG-14 by immunohistochemistry. These data confirm that TADG-14 protein expression is detectable on TADG-14 RNA-positive cervical cancer cells. Of interest, 100% of the primary squamous cervical carcinomas and 60% of the primary adenocarcino-

mas tested expressed TADG-14. These data suggest that both squamous cervical tumors and adenocarcinomas may commonly overexpress TADG-14. The value of TADG-14 as a diagnostic or therapeutic target may depend on differential expression between cancer and dysplasia. However, no data are yet available on TADG-14 expression by cervical intraepithelial lesions. Of interest, among other known proteases, TADG-14 most closely resembles the mouse protease known as neuropsin, which was originally cloned from mouse hippocampus and, subsequently, implicated in neuronal plasticity.¹⁹ If TADG-14 functions in a manner similar to mouse neuropsin, it may be capable of restructuring the three-dimensional architecture of a tumor, allowing for the shedding of tumor cells or the invasion of normal host tissues by degrading fibronectin.²⁰

Because in previous studies we were unable to detect the TADG-14 transcript in any of 50 normal human tissues studied, although after prolonged Northern blot exposure, extremely low levels of TADG-14 were detected in normal kidney, breast, and lung,⁸ our results suggest that this gene is under the control of a promoter that is most active in human tumors, including ovarian and cervical tumors, and that it may be possible to exploit this finding for diagnostic and/or therapeutic means. Consistent with this view, immunohistochemical staining of ovarian⁸ and cervical tumors (this article) revealed that TADG-14 is highly associated with tumor cells. Furthermore, because TADG-14 expression has also been reported in other types of cancer, including prostate, breast, and colon, it may prove to be an important

target for the inhibition of tumor progression in a variety of human tumors.

Persistent or recurrent cervical cancer no longer amenable to control with surgery or radiation therapy remains a discouraging clinical entity with a 1-year survival rate between 10% and 15%.²¹ However, in patients in whom recurrence is limited to the central pelvis, and in patients who have not previously received radiotherapy as primary or adjunctive treatment, cure is potentially achievable with ultraradical (ie, exenterative) surgery or radiation therapy, respectively. Because of the tendency of cervical cancer to directly invade adjacent deep pelvic tissues and spread by lymphatics, accurate assessment of disease status remains problematic. This is especially true in previously irradiated patients, in whom the distinction between radiation fibrosis and disease recurrence may be extremely difficult. A serum tumor marker that accurately reflects the active tumor status of the majority of patients with cervical cancer would be particularly useful in this setting. Because TADG-14 appears only in abundance in tumor tissue and TADG-14 contains a secretion signal sequence and immunohistochemical data suggest that TADG-14 is secreted,^{8,22} we speculate that this protease may prove to be a useful tool for the early detection of recurrent/persistent cervical cancer. Consistent with this view, PSA has already been validated as a tumor marker for the early diagnosis of prostate cancer because of its abnormal prevalence in the peripheral blood of patients.¹⁴ To validate this hypothesis, an enzyme-linked immunosorbent assay detection kit for quantification of TADG-14 in the circulation of ovarian and cervical cancer patients is under development in our laboratory. Future studies, however, will be necessary to evaluate the potential of TADG-14 as a novel tumor marker in cervical cancer.

The recognition of tumor antigen-loaded dendritic cells (DCs) as one of the most promising approaches to induce a tumor-specific immune response in vivo has recently generated widespread interest in the use of these "natural adjuvants" for the therapy of several human malignancies refractory to standard treatment modalities.²³ The identification and cloning of a group of preferentially expressed serine proteases, including TADG-14 as novel tumor-associated antigens, may therefore offer the opportunity to use these targets to assess the potential for therapeutic DC vaccination for the treatment of human cancer patients refractory to standard treatment modalities.²⁴ In agreement with this view, our laboratory has recently identified cytotoxic T lymphocyte epitopes against other serine proteases, including hepsin and stratum corneum chymotryptic enzyme, that are highly differentially expressed genes in ovarian carcinomas and pancreatic cancer.²⁴ These data, combined with the recently reported immunodominant role played by the serine protease PSA in the prostate

cancer-specific immune response induced by DCs transfected with amplified tumor RNA²⁵ or loaded with killed prostate cancer cells,²⁶ further suggest that peptide epitopes derived from TADG-14 may become selective targets for DC-driven CD8⁺ cytotoxic T lymphocyte against cervical cancer.

In conclusion, we have shown that primary and established cervical cancer cell lines, but not normal cervical epithelial cells, frequently overexpress the TADG-14 gene, and we have further demonstrated that cervical tumors expressed this transmembrane serine protease. On the basis of these findings, we suggest that TADG-14 has the potential to become a useful diagnostic tool for monitoring response to therapy as well as to detect early recurrence after treatment and may also serve as a novel target antigen for the therapy of cervical tumors refractory to standard treatment modalities.

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