The Novel Serine Protease Tumor-Associated Differentially Expressed Gene–15 (*Matriptase/MT-SP1*) Is Highly Overexpressed in Cervical Carcinoma

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BACKGROUND. Tumor-associated differentially expressed gene–15 (*TADG-15/matriptase/MT-SP1*) is a novel transmembrane serine protease involved in numerous biologic processes, including activation of growth and angiogenic factors and degradation of extracellular matrix components. To assess the value of TADG-15 as a possible marker for tumor detection and/or as a target for therapeutic intervention, the authors investigated the frequency of expression of TADG-15 in human cervical tumors.

METHODS. TADG-15 expression was evaluated in 19 cervical carcinoma cell lines (i.e., 11 primary tumor cell lines and 8 established cell lines) and in 8 normal cervical keratinocyte control cultures using reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, to validate gene expression data at the protein level, TADG-15 expression was evaluated by immunohistochemistry on paraffin embedded tissue from which all 11 primary tumor cell lines were established.

RESULTS. TADG-15 was expressed at high levels in 8 of 11 (73%) primary cervical carcinoma cell lines and in 6 of 8 (75%) established cervical carcinoma cell lines by RT-PCR. Expression of TADG-15 was found in 6 of 6 (100%) primary squamous cell cervical carcinomas, whereas 2 of 5 (40%) primary adenocarcinomas expressed TADG-15. In contrast, none of the normal cervical keratinocyte control cultures ($n = 4$) or flash-frozen normal cervical biopsy specimens ($n = 4$) expressed TADG-15. Immunohistochemistry staining of paraffin embedded cervical carcinoma specimens confirmed TADG-15 expression in tumor cells and its absence on normal cervical epithelial cells.

CONCLUSIONS. Cervical carcinoma cells expressed high levels of TADG-15, suggesting that this protease may play an important role in invasion and metastasis. Because TADG-15 appears only in abundance in squamous tumor tissue and contains a proteolytic cleavage site, suggesting that the TADG-15 protease domain is released, it may prove to be a useful diagnostic tool for the early detection of recurrent/persistent cervical carcinoma after standard treatment or as a novel molecular target for therapy in patients with cervical carcinoma. ***Cancer* 2003;98: 1898–904.** © *2003 American Cancer Society.*

KEYWORDS: cervical carcinoma, tumor-associated differentially expressed gene–15, matriptase, serine protease.

High levels of proteolytic activity have been implicated in neoplastic progression. Degradation of the extracellular matrix (ECM), including the basement membrane and interstitial stroma, is required for metastatic tumor cells to migrate through anatomic barriers and to invade distant tissues.[1–3] Among the wide array of extracellular matrix-degrading proteases discovered in the last few years, serine proteases constitute a novel family of enzymes involved in numerous

biologic processes, including activation of complement, blood coagulation, activation of growth and angiogenic factors, and degradation of ECM components.[4,5] It is known that serine proteases contain conserved histidine, aspartate, and serine residues that are necessary for enzymatic activity and are either secreted enzymes or transmembrane enzymes awaiting cell surface release.[4] In addition, it has been reported that several membrane-spanning serine proteases have cytoplasmic N-terminal domains, suggesting possible functions in intracellular signal transduction.[4] Because a high level of serine protease expression in several human solid tumors compared with normal control cells has been demonstrated, these enzymes recently have been proposed as potential diagnostic, prognostic, and/or therapeutic targets for several human tumors.[5–11] Consistent with this view, two members of the serine protease family, prostate-specific antigen (PSA) and human kallikrein 2, already serve important clinical functions as prostate carcinoma biomarkers,[5] whereas other serine proteases (i.e., kallikrein 6, kallikrein 10, kallikrein 11, and kallikrein 14) are in the process of being validated as novel biomarkers for ovarian carcinoma.[8–12]

Tumor-associated differentially expressed gene–15 (TADG-15; *matriptase/MT-SP1*) is a trypsin-like serine protease with a cytoplasmic domain; a transmembrane domain with a cleavage site for release of extracellular domains; four low-density lipoprotein receptor-like, ligand-binding domains; and a serine protease domain.[13] The ligand-binding and serine protease domains all are extracellular and are released after proteolytic cleavage. Initially isolated by Shi et al.[14] as a novel protease expressed by human breast carcinoma cells, TADG-15 now has been cloned, and it has been shown that TADG-15 functions as an epithelial membrane activator for other proteases and latent growth factors.[15–18] Consistent with this view, it has been shown that TADG-15 activates the latent form of hepatocyte growth factor (HGF) to produce the active growth and motility factor that can bind to and activate the c-Met receptor.[16] TADG-15 has also been characterized as an in vitro activator of the calcium-regulating, protease-activated receptor-2 (PAR-2) as well as of the serine protease urokinase-type plasminogen activator (uPA), linking the latter to the activation of other protease systems important for tumor cell invasion and metastases.[16,17]

Our group recently developed a screening strategy using redundant primers to the conserved catalytic triad domain of the serine protease family. Using this approach to display serine protease transcripts that are found in abundance in gynecologic tumor tissues but that have little or no expression in normal tissues, we recently reported the identification and cloning of several transmembrane serine proteases, including TADG-15, in ovarian carcinoma.[18–22] In the current study, with the goal of assessing the value of serine proteases as possible markers for tumor detection and/or targets for therapeutic intervention, we have evaluated the extent and frequency of expression of the TADG-15 gene in several primary and established squamous and adenocarcinoma cervical carcinoma cell lines. To our knowledge, we report for the first time the expression of TADG-15 in the majority of cervical tumors evaluated. These findings may have important implications for the use of TADG-15 protease as a diagnostic tool or as a molecular target for therapy in patients with cervical carcinoma.

TABLE 1
Patient Characteristics

Patient	Age (yrs)	Race	Disease stage	HPV type	Treatment
CVX-1	40	White	IB	16	Surgery
CVX-2	26	White	IB	16	Surgery
CVX-3	47	African American	IB	16	Radiotherapy
CVX-4	38	African American	IB	16	Surgery
CVX-5	40	White	IIA	16	Surgery
CVX-6	42	White	IB	18	Surgery
ADX-1	33	White	IB	18	Surgery
ADX-2	33	African American	IB	18	Surgery
ADX-3	27	White	IB	18	Surgery
ADX-4	50	African American	IB	18	Surgery
ADX-5	46	White	IB	18	Surgery

HPV: human papillomavirus; CVX: squamous cell cervical carcinoma; ADX: adenocarcinoma.

MATERIALS AND METHODS

Primary and Established Cervical Carcinoma Cell Lines

Nineteen cervical carcinoma cell lines (11 primary tumor cell lines and 8 established cell lines) were evaluated for TADG-15 expression by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Established, commercially available cervical carcinoma cell lines (i.e., CaSki, SiHa, MS-751, HT-3, C33a, Me180, HeLa, and C4-I) were purchased from the American Type Culture Collection. Tumors derived from primary specimens were staged according to the International Federation of Gynecology and Obstetrics operative staging system. Fresh tumor biopsy specimens from patients who were diagnosed with frankly invasive, Stage IB–IIA cervical carcinoma were obtained at the time of surgery through the Gynecologic Oncology Division and the Pathology Department at the University of Arkansas for Medical Sciences (UAMS) under approval of the Institutional Review Board. Patient characteristics are described in Table 1.

All but one patient underwent radical abdominal hysterectomy and lymph node dissection for invasive cervical carcinoma. Eight normal keratinocyte control samples (i.e., four primary keratinocyte cultures and four flash-frozen biopsy specimens) were obtained from cervical biopsies of hysterectomy specimens from women who were diagnosed with benign disease and had a previous report of a normal cytologic evaluation. Cervical carcinoma cell lines and normal keratinocyte cell lines were established by following previously reported, standard tissue culture techniques.[23] We studied 11 primary cervical carcinoma cell lines and 4 primary keratinocyte cell lines: Some had been cultured for 3–4 weeks, and others had been cultured for a much longer time. The purity of fresh tumor cultures was tested by morphology, immunohistochemistry staining, and/or flow cytometry with antibodies against cytokeratins. Only cell lines that contained $>$ 99% tumor cells were evaluated. Because tissue digestion and prolonged in vitro cell culture potentially may alter antigen expression, four adjunctive normal cervical keratinocyte biopsy specimens also were evaluated as negative controls for TADG-15 expression before enzymatic digestion and/or in vitro culture (i.e., they were snap frozen in liquid nitrogen immediately after collection).

RNA Isolation and cDNA Synthesis

RNA isolation from different cell lines was performed using TRIzol Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. To verify integrity, 4 μg of RNA from each sample were run in 1% agarose gels using 18S+28S Ribosomal RNA (Sigma, St. Louis, MO) as a positive control. First-strand cDNA was synthesized using 5 μg of total RNA, 1 $\times$ RT-PCR buffer, 5 mM $MgCl_2$, 1 mM dNTPs, 2.5 μM random hexamers, 1 unit (U)/μL Rnase inhibitor, and 2.5 U/μL MulLV Reverse transcriptase (GeneAmp RNA PCR kit; Applied Biosystems, Foster City, CA) in a total volume of 20 μL. RNA extracted from the CaOV3 serous papillary ovarian carcinoma cell line, previously reported to express TADG-15,[18] was used as a positive control.

PCR Analysis

The PCR reaction mixture consisted of cDNA, 0.4 μM of sense and antisense primers, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.025 U/μL Taq polymerase (GeneAmp 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 targets 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-15-specific primers that produced the 150–base pair (bp) product were as follows: 5′-ATGACAGAGGATTCAGGTAC-3′ forward and 5′-GAAGGTGAAGTCATTGAAGA-3′ reverse. The sequences of β-tubulin primers that produced 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 white New Zealand rabbits with poly-lysine–linked, multiple-antigen peptides derived from the amino acid sequence of TADG-15 (LFRDWIKENTGV), as reported previously.[18] Formalin fixed, paraffin embedded tissue blocks from 8 normal uterine cervices (i.e., controls) and from 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 UAMS and evaluated for TADG-15 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 using a Vectastain Elite avidin-biotin complex (ABC) Kit (Vector Laboratories, Burlingame, CA). Briefly, formalin fixed and paraffin embedded specimens were deparaffinized and processed routinely using microwave heat treatment in 0.01 M sodium citrate buffer, pH 6.0. The specimens were incubated in methanol with 0.3% H_2O_2 for 30 minutes at room temperature and then incubated with normal goat serum for 30 minutes. The samples were incubated with anti-TADG-15 peptide-derived polyclonal antibody for 1 hour at room temperature in a moisture chamber. This was followed by incubation with biotinylated antirabbit immunoglobulin G for 30 minutes, then incubation with ABC reagent for 30 minutes. The final products were visualized using the AEC substrate system (Dako, Carpenteria, CA), 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 using 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 < 0.05.

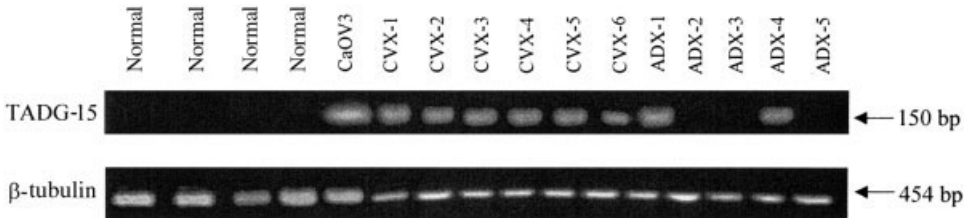

FIGURE 1. Tumor-associated differentially expressed gene–15 (*TADG-15*) expression was determined by reverse transcriptase-polymerase chain reaction analysis of primary squamous cell carcinoma (CVX1–CVX6), primary adenocarcinoma (ADX1–ADX5), and normal cervical keratinocyte primary cell lines. The reaction products were electrophoresed through a 2% agarose gel and stained with ethidium bromide. The 454-base pair (bp) band represents the *β-tubulin* product, and the 150-bp band represents the TADG-15 product. TADG-15 mRNA expression was not detected in any of the 4 normal cervical keratinocyte cell lines tested. In contrast, 6 of 6 (100%) squamous cell carcinoma cell lines and 2 of 5 (40%) cervical adenocarcinoma cell lines consistently expressed TADG-15.

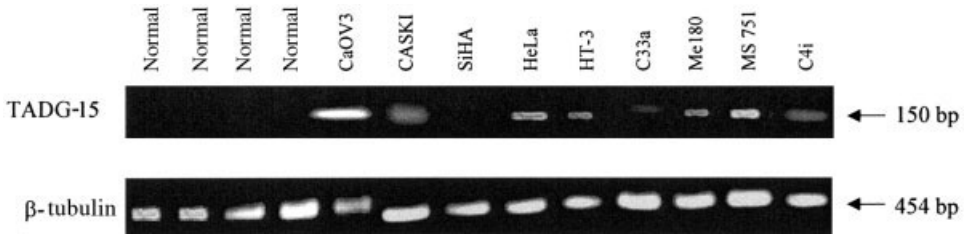

FIGURE 2. Tumor-associated differentially expressed gene–15 (*TADG-15*) expression was determined by reverse transcriptase-polymerase chain reaction analysis on established cervical carcinoma cell lines and normal keratinocyte flash-frozen biopsy specimens. The reaction products were electrophoresed through a 2% agarose gel and stained with ethidium bromide. The 454−base pair (bp) band represents the *β-tubulin* product, and the 150 bp band represents the TADG-15 product. TADG-15 mRNA expression was not detected in any of the 4 normal cervical keratinocyte samples tested. In contrast, 6 of 8 (75%) established cell lines expressed TADG-15.

RESULTS

TADG-15 Expression in Primary and Established Cervical Carcinoma Cell Lines

To characterize the frequency of expression of the TADG-15 gene in cervical tumors, we used RT-PCR with cDNA derived from primary and established squamous and adenocarcinoma cervical carcinoma cell lines as a template. Cultures of normal cervical keratinocytes were used as controls. PCR primers that amplify a TADG-15-specific, 150 bp product were synthesized and used in reactions. Primers that produce a specific, 454 bp PCR product for β-tubulin also were used as internal controls. Of the 19 cervical carcinomas studied, 15 carcinomas (79%) expressed TADG-15 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 carcinoma cell lines, and Figure 2 shows the RT-PCR results for the established cervical carcinoma cell lines. Both are representative of the typical results observed. Flash-frozen samples or cultures of normal cervical keratinocytes did not express TADG-15 (Figs. 1, 2). In contrast, TADG-15 was expressed in 8 of 11 (73%) primary cervical carcinoma cell lines ($P < 0.01$) and in 7 of 8 (87%) established cervical carcinoma cell lines ($P < 0.01$). For individual histologic subtypes of primary tumors, TADG-15 expression by primary squamous cell cervical carcinomas was found in 6 of 6 cell lines (100%), whereas 2 of 5 adenocarcinoma cell lines (40%) expressed TADG-15.

Immunohistochemical Staining

To determine whether TADG-15 expression detected by RT-PCR in primary cervical carcinoma cell lines was the result of the selection of a subpopulation of tumor cells present in the original tumor or whether in vitro expansion conditions may have modified gene expression, we performed immunohistochemical analysis of TADG-15 protein expression on formalin-fixed, paraffin-embedded tumor tissues from all primary tumors from which primary cell lines were derived. Table 2 shows that immunohistochemical staining supported the data obtained by RT-PCR. Using a TADG-15 peptide-directed antibody, we observed no significant staining with normal cervical keratinocytes samples (Fig. 3A). In contrast, intense staining was associated with cervical tumor cells of both adenocarcinoma and squamous histologic subtypes (Fig. 3C,D). Both adenocarcinomas and squamous cell carcinomas (Fig. 3C,D, respectively), similar to ovarian carcinoma cells (i.e., positive controls) (Fig. 3B), showed diffuse cytoplasmic staining for TADG-15.

TABLE 2
Tumor-Associated Differentially Expressed Gene–15 Expression in Squamous Cell Carcinoma and Adenocarcinoma of the Cervix and Normal Keratinocytes as Determined by Immunohistochemistry

Tissue type	TADG-15 expression
CVX-1	Positive
CVX-2	Positive
CVX-3	Positive
CVX-4	Positive
CVX-5	Positive
CVX-6	Positive
ADX-1	Positive
ADX-2	Negative
ADX-3	Negative
ADX-4	Positive
ADX-5	Negative
KRT-1	Negative
KRT-2	Negative
KRT-3	Negative
KRT-4	Negative
KRT-5	Negative
KRT-6	Negative
KRT-7	Negative
KRT-8	Negative

TADG-15: tumor-associated differentially expressed gene–15; CVX: cervical squamous cell carcinoma; ADX: adenocarcinoma; KRT: normal keratinocytes.

DISCUSSION

The activity of proteolytic enzymes is required at multiple stages during the growth, invasion, and progression of human tumors. Consistent with this view, several reports have demonstrated a positive correlation between the progression of malignant disease and the expression of extracellular proteases, such as matrix metalloproteinases (MMPs) and serine proteases (uPA), in a variety of human tumors.[1–5] Furthermore, it has been shown that inhibitors of these proteolytic enzymes interfere with the spread of malignancy in animal models.[24] These findings have prompted efforts to develop novel antitumor therapies directed against the proteolytic cascade initiated by MMPs and serine proteases and the various signaling pathways mediated by these enzymes.

Recently, the organization of the serine protease gene family (i.e., the kallikrein family) has been elucidated fully.[5] This family contains 15 genes that encode secreted serine proteases, which share significant homologies at both the DNA level and the amino-acid level. It is noteworthy that several members of this family reportedly are expressed highly differentially in human malignancies, including breast, ovarian, and prostate carcinomas, compared with normal tissue control cells.[5–15] Among these proteases, TADG-15 has received much attention because of its ability to cleave and activate uPA, the protease-activated receptor-2 (PAR-2), and the HGF activator (HGF/SF).[16,17] Many studies have implicated uPA, PAR-2, and HGF in the metastatic process, in angiogenesis, and in the growth and motility of various human tumors.[16,17,25–27] Thus, TADG-15 may play a crucial role in the growth and/or invasion of human malignancies through its activation of pro-uPA, pro-PAR-2, and pro-HGF. In a finding consistent with this view, we recently identified overexpression of TADG-15 and other secreted proteases in a high percentage of ovarian carcinomas.[18–22] Because of the paucity of information on serine protease expression in cervical carcinomas, in this work, we have analyzed the expression of the serine protease TADG-15 in several short-term primary cultures as well as established cervical carcinoma cell lines. In this pilot work, we chose this approach to evaluate differential TADG-15 gene expression in highly enriched populations of cervical tumor-derived epithelial cells. We report for the first time that TADG-15 was expressed in 15 of 19 (79%) cervical carcinomas studied, whereas it was undetectable in normal cervical keratinocytes. Therefore, these data support the view that TADG-15 is a serine protease that is expressed highly differentially in cervical tumors compared with normal control cells (i.e., cervical keratinocytes). Furthermore, to validate gene expression data at the protein expression level, TADG-15 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-15 expression was detected by RT-PCR expressed detectable TADG-15 by immunohistochemistry. These data confirm that TADG-15 is expressed in TADG-15 RNA positive cervical carcinoma cells. It is noteworthy that 100% of the primary squamous cervical carcinomas and 40% of the primary adenocarcinomas tested positive for TADG-15. Thus, these data suggest that the majority of squamous cervical tumors and a significant number of adenocarcinomas may overexpress TADG-15. The value of TADG-15 as a diagnostic or therapeutic target may depend on differential expression between different stages of cervical carcinoma and dysplasia. However, to date, no data are available on TADG-15 expression by cervical intraepithelial lesions.

Although surgery and radiation therapy represent effective treatment modalities for patients with cervical carcinoma, up to 35% of patients overall will develop recurrent, metastatic disease, for which treatment results remain poor.[28] However, in patients who have recurrent disease that is limited to the central pelvis, and in patients who have not previously received radiotherapy as primary or adjunctive treatment, cure potentially is achievable with ultraradical

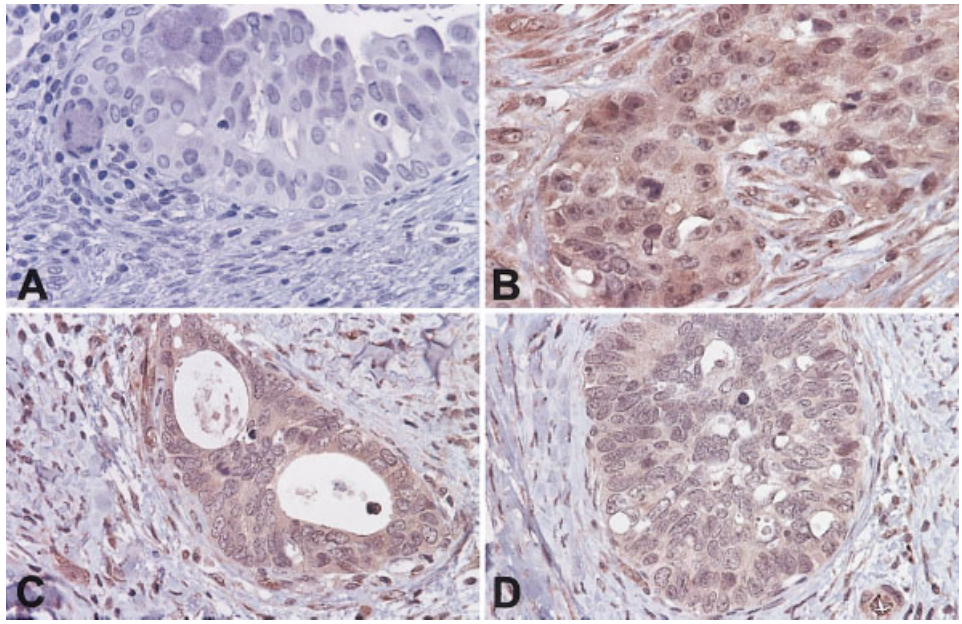

FIGURE 3. Immunohistochemical analysis of tumor-associated differentially expressed gene–15 (TADG-15) expression in cervical tumors. Representative immunoreactivity for TADG-15 in (A) normal epithelial cells, (B) ovarian serous papillary carcinomas (positive control), (C) adenocarcinoma ADX-1 cells, and (D) cervical squamous cell carcinoma CVX-2 cells. Although no staining was observed in normal cervical keratinocytes, in both squamous cell carcinoma and adenocarcinomas, TADG-15 exhibited diffuse staining throughout all tumor cells. Original magnification ×400.

(i.e., exenterative) surgery or radiation therapy, respectively. Because of the tendency of cervical carcinoma to invade adjacent deep pelvic tissues directly and to 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 carcinoma would be particularly useful in this setting. Because TADG-15 appears in abundance in squamous cell carcinoma, and because TADG-15 can be released, as suggested by immunohistochemical data,[13–15,18] we speculate that this protease may prove to be a useful tool for the early detection of recurrent and/or persistent cervical carcinoma. Consistent with this view, PSA and kallikrein 2 already have been validated as tumor markers for the early diagnosis of prostate carcinoma due to their abnormal prevalence in the peripheral blood of patients;[5] whereas other secreted members of the serine proteases family including kallikrein 6, kallikrein 10, kallikrein 11, and kallikrein 14, are in the process of being validated as novel biomarkers in other human solid tumors.[8–12] To validate this hypothesis, an enzyme-linked immunosorbent assay detection kit for quantification of TADG-15 in the circulation of patients with ovarian and cervical carcinoma is under development in our laboratory. However, future studies will be necessary to evaluate the potential of TADG-15 as a novel tumor marker in cervical carcinoma.

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 recently has generated widespread interest in the use of these 'natural adjuvants' for the treatment of several human malignancies that are refractory to standard treatment modalities.[29] The identification and cloning of a group of preferentially expressed serine proteases, including TADG-15, as novel tumor-associated antigens, therefore, may offer the opportunity to use these targets to assess the potential of therapeutic DC vaccination for the treatment of patients with malignant disease that is refractory to standard treatment modalities.[30] In a finding concordant with this view, our laboratory recently has identified cytotoxic T lymphocyte (CTL) epitopes against some serine proteases, including hepsin and kallikrein 7 (SCCE), genes that are expressed highly differentially in ovarian carcinomas and pancreatic carcinoma.[30] These data, combined with the recently reported immunodominant role played by the serine protease PSA in the prostate carcinoma-specific immune response induced by DCs transfected with amplified tumor RNA[31] or loaded with killed prostate carcinoma cells,[32] further suggest that peptide epitopes derived from TADG-15 may become selective targets for DC-driven, CD8 positive CTLs against cervical carcinoma.

In conclusion, we have shown that 100% of primary squamous tumors of the uterine cervix and 40% of cervical adenocarcinoma cell lines, but not normal cervical epithelial cells, overexpress the TADG-15 gene. We have also demonstrated that these tumors expressed this transmembrane serine protease. On the basis of these findings, we suggest that TADG-15 has

the potential to become a useful diagnostic tool for monitoring response to therapy and for detecting early recurrences after treatment, and TADG-15 also may serve as a novel target antigen for the therapy of patients with cervical tumors that are refractory to standard treatment modalities. The future design and implementation of clinical trials ultimately will determine the validity of this approach.

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