

Epitope Recognition by Anti-cathepsin D Autoantibodies in Endometrial Cancer Patients

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Objective. This study analyzed a model for the identification of specific epitopes recognized by autologous tumor-reactive humoral responses of endometrial cancer patients as potential markers for the monitoring of cancer.

Methods. The presence of circulating pro- and mature forms of cathepsin D and antibodies reactive with this enzyme were identified by Western immunoblot and quantitated by an enzyme immunoassay. Specific immunoreactivities with 34- and 52-kDa cathepsin D forms were analyzed by Western immunoblot using sera from endometrial cancer patients ($n = 40$) and normal volunteers ($n = 15$). Subsequently, reactivities with specific cathepsin D epitopes were defined by a peptide-specific ELISA.

Results. Circulating pro-forms of cathepsin D were detected in 31 of 40 endometrial cancer patients tested and none of the control volunteers. Circulating IgG reactive with cathepsin D could be demonstrated in 29/31 patients with circulating procathepsin D, while an anti-cathepsin D response was not detectable in normal controls. This response appeared to be directed against the pro-peptide portion of cathepsin D. Using a peptide-specific ELISA, the frequencies of antibody production against specific epitopes within the pro-peptide were defined.

Conclusion. There is a demonstrable tumor-reactive immune response elicited in endometrial cancer patients, directed against specific antigenic epitopes, some of which are conserved among these patients. Since these proteins are recognized as non-self, due at least in part to posttranslational processing errors, defining these epitopes will be useful as a means of diagnosis, assessment of therapeutic success, and, ultimately, identification of immunotherapeutic targets. © 2001 Academic Press

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INTRODUCTION

While in most gynecologic cancers B cells exhibit impaired effector functions, immune recognition of tumor antigens has been unequivocally shown. Studies have indicated the presence of tumor-reactive immunoglobulins in cancer patients, including melanoma [1], breast [2, 3], endometrial [4], and ovarian

cancers [5–9]. Cancer patients frequently develop anti-tumor immune responses, both humoral and cellular, against antigens expressed by their tumors. These immune responses in cancer patients appear to be elicited by antigens as a result of aberrant gene or oncogene expression or as a consequence of posttranslational alterations, including changes in glycosylation or phosphorylation.

Early attempts to identify tumor-associated antigens relied upon the xenogeneic recognition of human tumor-derived antigens in mice, the production and expansion of reactive hybridomas, and the subsequent absorption against batteries of normal tissues. The primary antigens recognized by mice were highly immunogenic antigens, such as histocompatibility antigens. While a number of overexpressed or inappropriately expressed antigens were identified in this manner, including carcinoembryonic antigen and α -fetoprotein, the identification of antigens which were unique to cancer or that possess unique epitopes has remained elusive. The use of patients' "autoimmune" response to identify tumor antigens, in theory, eliminates the general recognition of self-antigens and focuses on aberrant epitopes, even on commonly expressed proteins.

Our recent studies, utilizing tumor-reactive immunoglobulins derived from the autologous response, have identified specific tumor-associated antigens and defined tumor-linked alterations in specific epitopes of commonly expressed antigens [10, 11]. One antigen identified in cancer patients was cathepsin D (pro- and mature forms). The presence of cathepsin D in these immune complexes further confirms the development of cathepsin-D-reactive antibodies in these patients.

The most important immediate features of the presence of tumor-reactive antibodies are their specificity for common aberrant epitopes and the timing of their appearance. Since tumor-reactive immunoglobulins are elicited and detectable prior to the development of a palpable tumor or circulating antigen or prior to the clinical manifestations of recurrence, the assessment of the tumor-reactive immune response against specific antigenic epitopes should represent an early diagnostic and prognostic marker, superior to those currently available. It is our hypothesis that some tumor-reactive immunoglobulins derived from the autologous response may recognize tumor-

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associated alterations in specific epitopes of commonly expressed antigens, such as procathepsin D. Since tumor-reactive antibodies appear prior to the detection of palpable tumor or circulating antigens, measurement of these antibodies and analysis of the recognized antigens can be utilized for early diagnostic assays and vaccine development.

MATERIALS AND METHODS

Patient materials. Sera were obtained from endometrial cancer patients in the Gynecologic Oncology Clinic and from normal volunteers from the Gynecology Clinic of the Department of Obstetrics of the University of Louisville School of Medicine under an informed consent protocol approved by the human studies committee. Blood from 40 endometrial cancer patients at various stages of disease and 15 normal (non-cancer-bearing), age-matched volunteers were collected in standard red-top collection tubes. Blood samples were allowed to clot and were then centrifuged at 400g for 10 min to sediment cells and clot. The serum was removed and stored in polycarbonate tubes at -20°C until analysis.

Presence of circulating forms of cathepsin D. To identify the forms of cathepsin D present in the circulation of endometrial cancer patients and normal controls, cathepsin D was immunoprecipitated from serum by incubating the serum (250 μl) with protein G-agarose (Sigma Chemical Co., St. Louis, MO) for 1 h to preclarify. After centrifugation at 3000 rpm, the supernatants were removed and incubated with affinity-purified anti-cathepsin D antibody (Calbiochem, San Diego, CA) coupled to agarose (20 μl) overnight. The agarose-bound complexes were isolated by centrifugation at 3000 rpm for 2 min. These isolated complexes were washed three times with PBS containing 1% Triton X-100. The complex was then boiled in SDS-PAGE sample buffer, containing 100 mM sucrose, 3% SDS, 62.5 mM Tris-HCl, pH 6.9, 2 mM EDTA, and 270 mM β -mercaptoethanol. Immunoprecipitated cathepsin D was then analyzed by Western immunoblot as described below.

Purification of 52- and 34-kDa cathepsin D. For studies examining patient-derived antibody response against cathepsin D, two forms of cathepsin D (34 and 52 kDa) were isolated from the serum of an endometrial cancer, overexpressing these two forms of cathepsin D, using affinity chromatography using pepstatin A (Sigma Chemical Co.) [15]. The eluted fractions were analyzed by Western immunoblot to distinguish the various forms of cathepsin D. Fractions containing the 34- and 52-kDa cathepsin D's were pooled and concentrated. The concentration of purified procathepsin D protein was determined by the Bradford Coomassie brilliant blue assay (Bio-Rad Laboratories, Richmond, CA) [12].

Electrophoresis and Western immunoblot analysis. The level and molecular forms of cathepsin D were assayed by Western immunoblot using monoclonal anti-cathepsin D as the primary antibody, while the presence of patient-derived anti-

cathepsin D was determined by Western blot using diluted patient serum as the source of primary antibody [13]. The immunoprecipitates or purified cathepsin D forms were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gel by the method of Laemmli [14] and electrophoretically transferred onto nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk and probed overnight at 4°C in the same buffer with either monoclonal anti-cathepsin D (Calbiochem) or sera from normal controls or endometrial cancer patients (diluted 1:100). After this incubation the membranes were washed three times in 0.1% Tween 20 in TBS for 15 min. The blots were then incubated in blocking buffer with either rabbit anti-mouse IgG (positive control with murine monoclonal) or goat anti-human immunoglobulins conjugated with horseradish peroxidase (Sigma Chemical Co.) for 45 min at room temperature. These immunoblots were washed three times in 0.1% Tween 20 in PBS, after which bound complexes were visualized by enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL).

Enzyme-linked immunosorbent assay (ELISA) for cathepsin-D-reactive antibodies. To quantitate the differences in the intensity of immunoreactivity observed in Western immunoblot, an ELISA technique assessed the binding of patient antibodies to endometrial tumor-derived cathepsin D. The pooled 34- and 52-kDa cathepsin D forms were equilibrated in coupling buffer consisting of 100 mM sodium carbonate and 0.5M NaCl, pH 8.3, and added to the wells of a 96-well MaxiSorp microtiter plate (Nalge-Nunc International, Naperville, IL). The plates were subsequently washed and then blocked with SuperBlock (Pierce Chemical Co., Rockford, IL) for 1 h. Sera from endometrial cancer patients and normal age-matched normal control women (both diluted 1/100) were added to quadruplicate wells. The plates were washed and incubated with peroxidase-conjugated anti-human IgG. After three washes, the level of bound antibody was determined by incubating the wells with ortho-phenylenediamine (OPD, 0.4 mg/ml) and reading the absorbance at 490 nm. Statistical comparisons were made between the relative absorbance for reactive IgG on the patients using the Mann-Whitney test.

Presence of antibodies reactive with the 34- and 52-kDa cathepsin D. To define the specific reactivity of patients directed toward cathepsin D, the presence of cathepsin-D-reactive antibodies was further assayed by Western immunoblot. The purified 34- and 52-kDa cathepsin D were pooled and separated by SDS-PAGE and processed for Western immunoblot as described above. The blots were probed overnight at 4°C with sera from normal controls or endometrial cancer patients (diluted 1:100).

Peptide-specific ELISA. To define specific epitopes recognized by the patients' anti-cathepsin D response, purified 52-kDa procathepsin D was subjected to proteolytic digestion with 5 ng of modified trypsin (Promega, Madison, WI). Proteolytic

digestion was allowed to proceed overnight at 37°C. The resulting solution of proteolytic peptides was then acidified with acetic acid to give a final acid concentration of 1% and reduced to dryness in a speedvac. These proteolytic digests were dissolved in 50 μ l of 100 mM ammonium acetate/50% acetonitrile, followed by the addition of 2 μ l of 100 mM DTT. After 20 min, 100 μ l of 100 mM methyl 4-nitrobenzene sulfonate was added and, after incubation at 37°C for an additional 40 min, the samples were brought to dryness. The pellets were redissolved in 25 μ l of 25% acetonitrile with 0.05% TFA and subsequently diluted with 110 μ l of 0.05% TFA. The samples were then fractionated by RP-HPLC on a Vydac C18 column (2.1 $\times$ 250 mm) using a 105-min discontinuous gradient of 0.06% TFA:99.94% water to 0.052% TFA:78.4% acetonitrile:21.5% water. The resulting peaks will be monitored by their absorbance at 214 nm.

Each resulting peptide was diluted 1/25 in coupling buffer and adhered to wells of a MaxiSorp microtiter plate, as described above. These plates were blocked and incubated with sera, diluted 1/100, or monoclonal anti-cathepsin D (1 μ g/ml). The plates were washed and incubated with peroxidase-conjugated secondary antibody. The presence of bound antibody was determined by incubating the wells with a 50 mM citrate buffer solution containing OPD (0.4 mg/ml). Peaks corresponding to the positive wells of the peptide-specific ELISA were subjected to amino acid microsequencing. To identify the commonly recognized antigenic epitopes, reactivities of four endometrial cancer patients were analyzed in a peptide-specific ELISA.

To determine the frequency of the epitope recognition, the peptide-specific ELISA was repeated using specific peptides, defined as being commonly recognized by cancer patients' immune responses. Specific tryptic peptides were coupled to wells of a 96-well MaxiSorp microtiter plate (Nalge-Nunc International). The plates were blocked and then incubated with patients' sera, diluted 1/100 as described above. The plates were washed and incubated with peroxidase-conjugated secondary antibody. The presence of bound antibody was determined by incubating the wells with a 50 mM citrate buffer solution containing OPD (0.4 mg/ml).

Statistical analysis. The reactive bands for expression of the pro- and mature forms of cathepsin D were quantitated by densitometry, with relative absorbances for these bands standardized to control lanes included on each gel. All relative absorbance determinations were performed at least twice by Kodak 1D Image Analysis software (Eastman Kodak Co., Rochester, NY) and the mean $\pm$ standard error of the mean for each sample was calculated. The quantitation of cathepsin-D-reactive antibodies in the total cathepsin D ELISA and the peptide-specific ELISA were determined in quadruplicate and means $\pm$ standard deviations were calculated. Statistical comparisons were made between these means for reactive IgG in patients and controls using the Mann-Whitney test. The com-

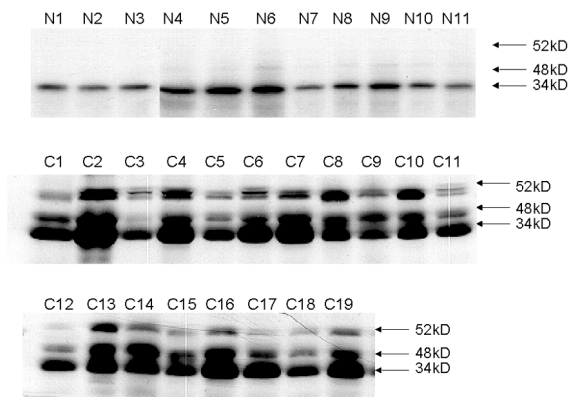


FIG. 1. Representative Western immunoblots demonstrating the presence of circulating 52-, 48-, and 34-kDa forms of cathepsin D in the sera of non-tumor-bearing controls and endometrial cancer patients. Cathepsin D was immunoprecipitated from 250 μ l of serum and visualized using monoclonal anti-cathepsin D. Non-cancer-bearing control female volunteers are designated N1–N11 and endometrial cancer patients are indicated as C1–C19.

parisons of reactive antibody positive and negative populations were performed using Fisher's exact test.

RESULTS

Circulating forms of cathepsin D. Our previous studies have demonstrated the presence of a humoral response against cathepsin D, indicating the exposure of aberrant cathepsin D to the immune system. Initially, to characterize this exposure, the levels and molecular weight forms of cathepsin D in the circulation were defined in normal volunteers and endometrial cancer patients (for representative samples, see Fig. 1). In addition to overexpression of the mature molecular weight form (34 kDa), circulating pro-forms of cathepsin D (48 and 52 kDa) were identified in 31/40 endometrial cancer patients, while in normal controls and the remaining 9 endometrial cancer patients only the 34 kDa was demonstrable.

Immunoreactivity of patient-derived IgG against cathepsin D. Tumor-reactive IgG has been previously demonstrated in these patients. To identify whether cathepsin-D-reactive antibodies resulted from the exposure of the patients' immune systems to pro-forms of the enzyme, the presence and levels of serum-derived cathepsin-D-reactive antibodies were assayed using an ELISA. The relative levels of anti-cathepsin D antibodies in the 31 endometrial cancer patients positive for circulating 48- and 52-kDa forms of cathepsin D were compared to those of normal non-tumor-bearing volunteers (Fig. 2). Significant levels of anti-cathepsin D antibodies were observed in 29/31 cancer patients (0.385 ± 0.144) and in none of the controls (0.042 ± 0.37) ($P < 0.0001$). In addition, none of the 9 endometrial cancer patients not expressing circulating pro-cathepsin exhibited antibodies against cathepsin D (data not shown).

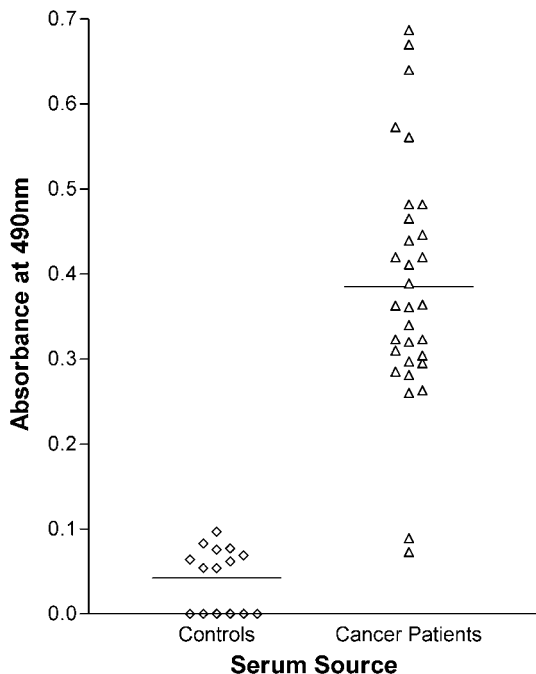


FIG. 2. ELISA quantitation of antibodies reactive with tumor-derived cathepsin D's in the sera of normal controls ($n = 15$) and endometrial cancer patients. The presented reactivity against cathepsin D was assayed in the 31 endometrial cancer patients positive for circulating cathepsin D pro-forms.

The presence of immunoglobulins specifically reactive with the pro- and mature (52 and 34 kDa) proteins purified from an endometrial cancer patient was further examined by Western immunoblot. The presence of these proteins was confirmed by comparison of patient immunoreactivity with commercial monoclonal anti-cathepsin D antibodies (representative data are shown in Fig. 3). Sera from normal controls failed to recognize either protein. In contrast, the sera from the endometrial cancer patients recognized only the 52-kDa procathepsin D.

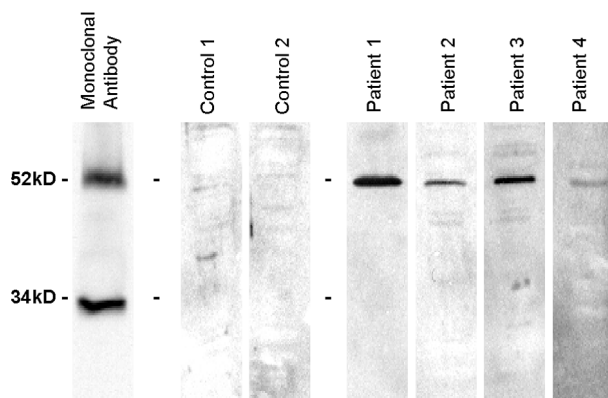


FIG. 3. Representative Western immunoblots demonstrating the immunoreactivity of antibodies in the sera of endometrial cancer patients with purified 34- and 52-kDa cathepsin D compared to the reactivity of an anti-cathepsin D monoclonal antibody.

TABLE 1
Amino Acid Sequence and Location within the Procathepsin Molecule of Peptides Commonly Recognized by Four Advanced-Stage Endometrial Cancer Patients, as Determined by Peptide-Specific ELISA

Designation	Amino acid number	Sequence	Location
Peptide 1	24–28	IPLHK	Pro-peptide
Peptide 2	50–70	YSQAVPAVTEGPIPEV	Pro-peptide

Note. Tryptic peptides were derived from procathepsin D. These peptides were separated by RP-HPLC and each peptide was coupled to wells of a MaxiSorp microtiter plate.

Identification of procathepsin D epitopes recognized by patient-derived IgG. Subsequently, to identify commonly recognized antigenic epitopes, immunoreactivity of the sera from four advanced-stage endometrial cancer patients expressing procathepsin D was examined in a peptide-specific ELISA. Two antigenic epitope-containing peptides were identified that were recognized by all four patients tested. These epitopes resided in the pro-peptide portion of the procathepsin D (Table 1). To determine the frequency of the recognition of each peptide, the peptide ELISA was performed using only these two procathepsin D peptides. Of the 29 patients producing antibodies reactive with procathepsin D, 20/29 ($P = 0.0007$ vs controls) recognized peptide 1 and 23/29 ($P < 0.0001$ vs controls) recognized peptide 2 (Table 2). None of the 10 control sera recognized these peptides.

DISCUSSION

The identification of new targets for diagnostic screening is a major focus of current cancer research. Circulating tumor-derived proteins, such as CA125, placental alkaline phosphatase, and cathepsin D, have been investigated as potential screening markers for ovarian cancer; however, since these antigens are also expressed by some normal tissues and can be overexpressed in certain noncancerous conditions, the available markers lack the specificity to be an effective screening

TABLE 2
Reactivity of Immunoglobulins Derived from Sera of Normal Controls and Endometrial Cancer Patients (Previously Demonstrated to Possess Procathepsin-D-Reactive Antibodies) with Specific Epitopes Contained in Peptides Derived from the Digestion of Procathepsin D with Modified Trypsin

Designation	Location	Reactivity with controls	Reactivity with patients
Peptide 1	a.a. 24–28 propeptide	0/10	20/29
Peptide 2	a.a. 50–70 propeptide	0/10	23/29

tool. Our previous work has established that certain tumor-associated antigens elicit a readily demonstrable autologous humoral response. Overexpression exclusively or expression within an inappropriate tissue is generally inadequate to elicit such an autologous response. These autologous immune responses appear to be elicited by "antigenic" sites resulting from mutations in the primary structure or from aberrant posttranslational modifications, including changes in glycosylation or phosphorylation.

In animal models, immune recognition of tumor antigens has been unequivocally shown; however, only recently has recognition of tumor-associated antigens been clearly demonstrated in cancer patients [16]. Our previous work has indicated that posttranslational processing errors, leading to overexpression and secretion of procathepsin D, could autoimmunize cancer patients [17]. While cathepsin D is a normal lysosomal enzyme and the mature enzyme can be detected in the sera of non-tumor-bearing individuals (Fig. 1), these processing errors, resulting in significant circulating levels of pro-form and the subsequent development of a humoral response, appear to be unique to cancer. Previously, Brouillet *et al.* [18] suggested that utilizing an immunoassay detecting circulating procathepsin D should enhance cancer specificity compared to total cathepsin D immunoassays currently used.

Our past work with cathepsin D has provided evidence as to the plausibility of developing an autologous antibody assay to this enzyme in an attempt to exploit it for future use in both diagnosis and treatment. The cathepsin D model appeared plausible for two reasons. First, previous work has shown that cathepsin D was associated with a wide variety of tumor types, including ovarian and endometrial. Second, we have previously sequenced the amino acid sequence of this enzyme, making manipulation for the development of the ELISA assay manageable. Thus, this study addressed the utility of the humoral response to define specific epitopes of the cathepsin D protein, which could be subsequently targeted for the development of diagnostic assays.

In the present study, endometrial cancer patients (31/40) generally expressed pro-forms of cathepsin D, while only the mature (34 kDa) form was detectable in the sera of non-tumor-bearing controls. Currently utilized total cathepsin D ELISAs do not distinguish the presence of the various molecular forms, but measure only the total amount of circulating enzyme. While the individuals in our study would have been identified by these assays as having overexpressed enzyme, there are other nonmalignant conditions that would also give positive results.

Of the 31 cancer patients exhibiting circulating pro-forms, 29 patients exhibited antibodies reactive with forms of cathepsin D (Fig. 2). Cathepsin-D-reactive antibodies could not be demonstrated in the 9 patients with endometrial cancer who did not exhibit circulating pro-cathepsin D or in the normal controls. Thus, it appeared that the enzyme-reactive antibodies were elicited by the presence of the pro-forms of cathepsin D.

When the presence of immunoglobulins reactive with the 52-kDa procathepsin D and 34-kDa mature enzyme (purified from an endometrial cancer patient) was subsequently examined by Western immunoblot, the antibodies from endometrial cancer patients recognized the 52-kDa procathepsin D, but not the mature enzyme (Fig. 3).

Since this immune response failed to recognize the mature enzyme, this recognition appeared to be directed toward epitopes expressed within the pro-peptide portion of procathepsin D. To identify any commonly recognized epitopes within the procathepsin D protein, a peptide-specific ELISA using all of the generated tryptic peptides was assayed with sera from four patients. Two peptides were identified by all four endometrial cancer patients, while none were identified by controls. Of the 29 patients producing antibodies reactive with procathepsin D, 69% recognized peptide 1 and 79% recognized peptide 2. The sera from non-tumor-bearing controls failed to recognize these peptides.

Measurement of circulating cathepsin D levels appears to be a useful marker for progression and response to therapy in a number of tumors, including endometrial cancer. The current assay systems measure total cathepsin D and do not distinguish pro-forms from mature forms. Some nonneoplastic conditions can result in elevated total cathepsin D; thus, this general measurement lacks specificity. For the production of an immune response, cathepsin D must be overexpressed and exhibit an alteration causing it to be recognized as foreign. Such alterations include mutations and altered posttranslational modification (i.e., aberrant glycosylation or phosphorylation or modified processing).

Rocheffort *et al.* [19] demonstrated that the overexpression and secretion of procathepsin D increase the incidence of clinical metastases by increasing cell growth and decreasing contact inhibition, rather than mediating the escape of cancer cells through the basement membrane as previously postulated. This dysregulated growth of tumor cells, as well as the development of chemoresistance, appears to be mediated by the pro-peptide portion of cathepsin D [20, 21]. Thus, the overexpression and altered posttranslational modifications of cathepsin D producing circulating pro-forms, observed in this study, can stimulate the malignant progression of tumors. In contrast to current assays for circulating cathepsin D, the presence of antibodies reactive with specific epitopes of procathepsin D is associated with not only the overexpression of cathepsin D, but also the presence the aberrant processing and modification of the pro-enzyme. Since immune responses generally precede detectable circulating antigen, the assessment of antibodies against tumor-derived antigens may be an earlier diagnostic marker than current assays measuring circulating antigen. The presence of antibodies recognizing specific epitopes may also be a prognostic marker, since it indicates the presence of the aberrant pro-peptide mediating increased cell proliferation and chemoresistance. This study specifically examined patient-derived antibody reactive with procathepsin D; however, the

approach described can be applied to any tumor-derived component eliciting an autologous humoral response. By identifying the presence of shared epitopes recognized by these reactive antibodies from ovarian cancer patients, we can define specific changes that are essential from neoplastic development and progression. By defining specific shared alterations in the procathepsin D molecule inducing the malignant progression of tumor cells, these epitopes may ultimately represent important therapeutic targets.

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