Advances in Brief

Posttranslational Truncation and Inactivation of Human E-Cadherin Distinguishes Prostate Cancer from Matched Normal Prostate

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Abstract

An essential event in the progression of adenocarcinoma is the loss of organized epithelial attachment (both to the basement membrane and to adjoining epithelial cells). The E-cadherin cell adhesion molecule has an established function in maintaining normal phenotype and tissue homeostasis, and loss of E-cadherin function has been implicated in tumorigenesis. Aberrations in E-cadherin are associated with prostate cancer progression; however, these aberrations are not simply a result of prodigious allelic loss. We have previously demonstrated a novel posttranslational truncation within the cytosolic domain of native M, 120,000 E-cadherin to a membrane-bound M, 97,000 species. We hypothesize that truncation of E-cadherin is an inactivating event that is significantly increased in localized prostate tumors and that it represents a novel molecular event that may distinguish prostate cancer from adjacent normal tissue. E-cadherin was characterized by Western blot analysis in matched normal and cancer tissue from 18 prostate cancer patients. Imaging and densitometry software were used to quantify the truncation of E-cadherin by measuring the ratio of M, 97,000 E-cadherin to M, 120,000 E-cadherin, which was significantly increased in the tumor aspect of the prostate gland. Herein, we report the first experiment comparing case-matched human normal and cancerous prostate tissue in the context of E-cadherin truncation.

Introduction

Aberrations in E-cadherin expression have been noted in malignant degeneration of prostate epithelium, metastasis, and decreased patient survival (1–6). Loss of E-cadherin function does not seem secondary to allelic loss, but rather to transcriptional repression of the gene (7–9). Homeostasis of the prostate gland is, in part, maintained by the interepithelial transmembrane adhesion glyco-protein E-cadherin. The mature form of E-cadherin is a M, 120,000 protein (E-cad120)3 that consists of an extracellular portion of five tandem repeats, the most distal of which is required for calcium-mediated homotypic binding between E-cadherin molecules from neighboring epithelial cells (10–13). E-cadherin also contains an intracellular portion that is responsible for linking E-cadherin to the actin cytoskeleton through α, β, and γ catenin that is necessary for the adhesive function of E-cadherin (11, 14–17). Studies from our laboratory using cell culture and in vivo models have demonstrated the role of E-cadherin-dependent aggregation in prostate and mammary epithelial survival. We have also demonstrated that the posttranslational cleavage of the native E-cad120 results in a novel membrane-bound M, 97,000 fragment (E-cad73) in cells destined to undergo apoptosis (18). This cleavage effectively removes the β-catenin binding domain, rendering the E-cadherin molecule functionless. This inactivating truncation of E-cadherin may then represent a novel mechanism by which E-cadherin function is abrogated in human prostate cancer. In this study, we evaluated whether this truncation occurs in human prostate tissue with subsequent loss of the β-catenin binding domain and to determine whether E-cadherin truncation relates to clinical prostate cancer progression.

Materials and Methods

Tissue Procurement. Patients with localized prostate cancer provided written informed consent in an Institutional Review Board approved study at the University of Michigan to have their prostate tissue evaluated in experimental studies. Fresh radical prostatectomy specimens were frozen in liquid nitrogen within 30 min after surgical excision. Histological confirmation of both tumor and normal regions of each prostate gland were confirmed by a genitourinary pathologist at the University of Michigan, and the corresponding tumor or normal region was excised before processing for protein analysis.

Protein Preparation and Western Blot Analysis. To prepare protein lysates from procured human tissue, the samples were homogenized and allowed to lyse for 1 h (on ice) in protein lysis buffer [50 mM Tris (pH 7.5), 120 mM NaCl, and 0.5% NP40] in combination with the following protease inhibitors: 40 μM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 200 μM orthovanadate. Lysates were centrifuged, and the supernatants were collected and quantitated in triplicate in a 96-well microtiter Bradford assay. All experimental samples were quantitated on the same microtiter plate against the same BSA standard curve. The absorbance was read using a dual mode (test filter at 595 nm and reference filter at 405 nm on a Dynatech Laboratories MRX Microplate reader using End Point Program version 1.1). Curve fitting was done using a linear power fit with tails. Protein (150 μg) from each tumor and normal pair was loaded and separated on 6% Tris/Glycine precast NOVEX gels and analyzed using the NOVEX and enhanced chemiluminescence (Amersham) detection systems as described previously (19).

Bradford Assay for Microtiter Plate

Densitometry. One-minute exposures of the Western blots from the matched tumor and normal prostate specimens were scanned at 300 dpi in gray scale format into Vista Scan. These files were then imported into NIH imaging, and densitometry of each E-cad120 and E-cad73 band was then performed. Western blot densitometry results of matched prostate cancer and normal prostate clinical specimens were analyzed by the Wilcoxon matched pairs test using Statistica software (StatSoft, Tulsa, OK). Stratification by stage or progression status was not possible due to limited variance of these parameters in the study cohort.

Antibodies. To detect the E-cad120 and E-cad73 forms of human E-cad, the HECD-1 monoclonal antibody (Zymed Laboratories, Inc.), which was raised against the extracellular domain of E-cad, was used. The E-cad cytoplasmic antibody 4A2 was a gift from Dr. Margaret Wheelock (University of Toledo, Toledo, OH). For β-catenin immunoprecipitations and Western blots, the CAT-5H10 antibody (Zymed Laboratories, Inc.) was used. The appropriate
horseradish peroxidase-conjugated antimonine and antirat secondary antibody was, respectively, obtained from Ameresco and Jackson ImmunoResearch Laboratories.

**Immunoprecipitation Experiments.** The case-matched tumor and normal tissue lysates were prepared and quantitated as described above. For the immunoprecipitation reactions, the above protein lysis buffer was used. The lysates were precleared with the protein G-Sepharose beads (Zymed Laboratories, Inc.) before the immunoprecipitation reactions, and beads were diluted with an equal volume of Tris buffered saline (0.1% Tween 20, pH 7.6)/2.5% milk before use. After preclearing, 500 μg of protein were equalized to 500 μl with the protein lysis buffer. Ten micrograms of β-catenin primary antibody were added and rotated at 4°C overnight. Protein G-conjugated Sepharose beads (120 μl blocked) were added and allowed to mix 90 min at 4°C. The beads were pelleted and washed four times with 500 μl of the protein lysis buffer. Thirty μl of twice reducing sample buffer was added to each pellet, and the samples were heated at 100°C for 5 min and separated using 6% Tris/Glycine precast NOVEX acrylamide gels.

**Results**

**Western Blot Analysis of E-cad<sup>97</sup> and E-cad<sup>120</sup> in Human Prostate Tissue.** Prior studies have associated apoptosis with the truncation of E-cadherin (18), but clinical prostate cancer specimens have not been previously evaluated for such E-cadherin processing. For this purpose, consecutive radical prostatectomy specimens were identified that had sufficient amounts of both normal and cancerous prostate tissue for protein analysis. Eighteen patients with Gleason scores 6–8 and pathological stage T2 and T3 prostate cancer, whose preoperative prostate-specific antigen ranged from 5–24 ng/ml, constituted the cohort for this analysis. To confirm that the accumulation of E-cad<sup>97</sup> did not result from protein degradation during lysate preparation, the human prostate cell line LNCaP, which expresses only E-cad<sup>120</sup>, was subjected to the same protein isolation protocol, with no visible generation of the truncated species (data not shown).

Results from Western blot analysis of the 18 matched normal tumor pairs are shown in Fig. 1. In all of the tumor samples there is significant accumulation of E-cad<sup>97</sup>. In the normal specimens, 6 samples were devoid of the truncated band whereas the remaining 12 samples exhibited, in most cases, moderate accumulation of E-cad<sup>97</sup>. To further characterize this apparent increase in E-cad<sup>97</sup> in the tumor relative to its case matched normal, densitometry was performed to quantitate band intensity. Then, the fraction of truncated E-cadherin was determined for both the tumor and normal samples for each patient. The net difference between the ratio of E-cad<sup>97</sup>:E-cad<sup>120</sup> in the tumor relative to its case-controlled normal emphasizes a relative increase of E-cad<sup>97</sup> in the cancerous phenotype as graphically illustrated in Fig. 2. The actual densitometric data from individual specimens and the differences in ratios between tumor tissue and matched normal tissue controls are given in Table 1. In only four patients was the ratio of E-cad<sup>97</sup>:E-cad<sup>120</sup> greater in the normal tissue as compared with its case-matched tumor. In the remaining 14 patients, there is an increase in the E-cad<sup>97</sup>:E-cad<sup>120</sup> ratio in tumor aspect of the prostate gland compared with case-matched normal. The mean ratio of E-cad<sup>97</sup>:E-cad<sup>120</sup> for the normal group was 0.50 and for the tumor was 0.76. For the 18 patients, distribution of the E-cad<sup>97</sup>:E-cad<sup>120</sup> ratio was not normal in nature, and, therefore, a nonparametric analysis was performed. The Wilcoxon matched pairs test established a significant increase in E-cad<sup>97</sup>:E-cad<sup>120</sup> ratio in the tumor relative to its case-controlled normal is a statistically significant event as determined by Wilcoxon matched pairs (P = 0.035).

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**Table 1 Densitometry of Western blot analysis of truncated and full-length E-cadherin in clinical prostate cancer specimens**

**Fig. 2.** Differences in E-cad<sup>97</sup>:E-cad<sup>120</sup> ratios between normal and cancerous human prostate. Those values generated that are greater than zero indicate an increase in the amount of E-cad<sup>97</sup> in the tumor compared with its case-matched normal. The mean ratio E-cad<sup>97</sup>:E-cad<sup>120</sup> for normal and tumor tissue are given. The increase in E-cad<sup>97</sup>:E-cad<sup>120</sup> ratio in the tumor relative to its case-controlled normal is a statistically significant event as determined by Wilcoxon matched pairs (P = 0.035).
The β-catenin binding domain of E-cadherin, which is essential for the adhesive function of E-cad, has been mapped to the residues 815–839 in the cytoplasmic tail (10) and in in vitro and in vivo studies has been shown to be removed following truncation of E-cad

To determine whether this occurs in human prostate cancer, we used coimmunoprecipitation to examine the loss of β-catenin binding in the truncated E-cad. When lysates from localized prostate cancer specimens were immunoprecipitated with a β-catenin monoclonal antibody and the immunoprecipitated products immunoblotted with the HEC1-1 antibody, which recognizes both E-cad and E-cad79, only E-cad120 coprecipitated with β-catenin (see Fig. 3). Even long exposures of the immunoblot failed to demonstrate the presence of E-cad79 in the β-catenin precipitates (data not shown). This experiment suggested that truncation of E-cad120 removed the β-catenin binding domain, resulting in a nonfunctional, truncated E-cad79 with a resultant decrease in the E-cad/β-catenin functional complex available for intercellular adhesion.

Discussion

There have been many studies linking a decrease in E-cadherin expression to an increased aggressiveness of prostate cancer. Demarzo et al. (1) evaluated 76 localized prostate cancers for E-cad expression using immunohistochemical staining with HEC1-1 antibody. In addition to finding a correlation with tumor grade, they found that decreased E-cad expression along with preoperative prostate-specific antigen and Gleason score were predictive of pathologic stage in a multivariate analysis. Others have correlated decreased expression of E-cadherin with survival (4, 5). Umbas et al. (4) analyzed 89 patients and, along with finding a correlation between aberrant E-cad expression to grade and stage, also found a significant decrease in survival for those patients. Others have illustrated the correlation between E-cadherin function and tumor invasion by blocking E-cadherin activity and thereby increasing invasive potential or by transfection of cell lines with functional E-cadherin and inhibiting invasive behavior (20, 21).

The aforementioned studies have examined E-cadherin expression by immunohistochemical analysis of processed tissue sections. However, immunohistochemistry using the HEC1-1 antibody cannot discriminate between the full-length E-cad and truncated E-cad79. We demonstrate in this study evidence that the cleavage of E-cadherin in prostate tissue samples is easily and clearly detectable by Western blot and that the cleavage of E-cad120 effectively removes the β-catenin binding domain. Removal of the β-catenin binding domain inactivates E-cadherin, rendering it incapable of participating in intercellular adhesion. On the basis of our coimmunoprecipitation data, which demonstrates the absence of truncated E-cad79 in the β-catenin functional complex, we believe that the presence of E-cad79 then is indicative of ineffectual E-cadherin.

When the 18 prostatectomy specimens were analyzed with Western blot, there was a variable but significant difference between the case-matched tumor and normal specimens with respect to the relative amount of cleaved E-cadherin. Wilcoxon matched pairs analysis confirms a relative increase in the truncation in the tumor as compared with its normal. These results are based on the direct comparison between normal and cancer tissue from the same specimen eliminating potential confounding effects of comparing tumor and normal from different patients. Although equal amounts of lysate were loaded for each sample, the actual percentage of epithelium may vary depending on the relative amount of stroma present. We, therefore, calculated a ratio of E-cad79/E-cad120 to establish a value that is independent from the epithelial and stromal distribution.

A key determinant in tumor progression is the ability of cancer cells to survive and proliferate in the absence of extracellular contact. In tumors of epithelial origin, the loss of adhesion dependence may arise through alterations of E-cadherin-mediated pathways. This laboratory has previously demonstrated the role of E-cadherin-dependent aggregation in prostate and mammary epithelial survival and the correlation of E-cadherin truncation and inactivation in cells destined to undergo apoptosis (13, 18). Thus, the question arises, does the truncation and inactivation of E-cadherin correlate with apoptosis and the malignant phenotype of prostate carcinoma? There are two possible scenarios: because of the correlation between E-cadherin truncation and apoptosis, the accumulation of E-cad79 may reflect an increase in apoptotic index in prostate tumor cells. This is supported by one study that has demonstrated a positive correlation between apoptosis and an increasing malignant potential in prostate cancer (22). In the second scenario, we postulate that the accumulation of E-cad79 truncation may reflect early apoptotic signaling events resulting from the breakdown of adhesive interactions. However, although these cells may be in a detached state they are unable to execute apoptosis because of downstream mutations or inactivating events. Therefore, the truncated species may be accumulating in tumor cells that are not undergoing apoptosis. Undoubtedly, further analysis that will delineate the apoptotic and proliferative indices will help answer these questions.

Our study has used frozen tissue that was histologically confirmed as either normal prostatic tissue or tumor to analyze E-cad processing on the protein level. We have described a functional aberration in E-cad as a consequence of cleavage with subsequent loss of the β-catenin binding motif. As can be appreciated from the Western analysis of the 18 matched tumor normal pairs, there is a statistically significant increase in the ratio of the truncated E-cad79 to the native E-cad120 in the tumor relative to its case-matched normal control. The relative loss of β-catenin binding domain in the tumor, as demonstrated by increased accumulation of E-cad79 that is not present in the functional complex with β-catenin, provides an alternative mechanism for the loss of E-cadherin function.

It is prudent to restate that inactivation of E-cadherin in prostate cancer does result from prodigious allelic loss (7). Disruption of E-cadherin transcription does represent one mechanism by which E-cadherin is inactivated in this disease (7–9). Ablative transnational regulation and possibly posttranslational events have also been proposed as alternative mechanisms of E-cadherin dysfunction, but these possibilities have not previously been evaluated in clinical prostate cancer. Our findings indicate that the posttranslational truncation of E-cadherin is a novel and readily attainable end point for evaluating
loss of E-cadherin function that is significantly associated with the malignant transformation of the human prostate.

Acknowledgments

We thank Kathleen Day and Xin Zhao for expert technical assistance.

References

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