

Loss of Annexin 1 Correlates with Early Onset of Tumorigenesis in Esophageal and Prostate Carcinoma

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Abstract

Annexin I protein expression was evaluated in patient-matched longitudinal study sets of laser capture microdissected normal, premalignant, and invasive epithelium from human esophageal squamous cell cancer and prostatic adenocarcinoma. In 25 esophageal cases (20 by Western blot and 5 by immunohistochemistry) and 17 prostate cases (3 by Western blot and 14 by immunohistochemistry), both tumor types showed either complete loss or a dramatic reduction in the level of annexin I protein expression compared with patient-matched normal epithelium ($P \leq 0.05$). Moreover, by using Western blot analysis of laser capture microdissected, patient-matched longitudinal study sets of both tumor types, the loss of protein expression occurred in premalignant lesions. Concordance of this result with immunohistochemical analysis suggests that annexin I may be an essential component for maintenance of the normal epithelial phenotype. Additional studies investigating the mechanism(s) and functional consequences of annexin I protein loss in tumor cells are warranted.

Introduction

The development of rational approaches to the diagnosis and treatment of cancer is dependent on identifying and understanding the molecular mechanisms that underlie tumor formation and progression. In this regard, discovery-oriented studies that seek to determine the genes and proteins that are dysregulated in neoplasms are critical. The success of the Human Genome Project and several related gene discovery initiatives are facilitating these efforts as a tremendous number of new genes and proteins are available for study. However, at present, the expression profiles of the majority of genes are not known, and the expression status of only a small number of genes and proteins have been evaluated in human tumors. Thus, research efforts aimed at systematically identifying the genomic alterations, and gene and proteomic expression profiles of normal and tumor cells are critically needed. Genome-based investigations permit mutation analysis of specific genes as well as identification of genomic regions that are amplified or deleted in tumors. Studies at the mRNA level are capable of rapidly assessing the expression profiles of a large number of transcripts (1–3). Proteomic methodologies can identify quantitative and qualitative protein changes associated with malignant phenotypes, and can also be used to examine important posttranslational modifications such as the glycosylation or phosphorylation states of individual proteins (4–6).

Received 3/28/00; accepted 9/29/00.

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In the present study, we evaluated the expression of annexin I protein in two disparate tumor types: squamous cell carcinoma of the esophagus and prostate adenocarcinoma. Annexin I was selected for study based on the intersection of three earlier global molecular profiling studies that had been performed in our group using LCM² to acquire patient-matched normal and tumor epithelium from human tissue specimens. First, a high rate of DNA deletion was observed in esophageal tumors on chromosomal arm 9q near the *annexin I* gene (7), indicating a possible role for loss of gene function in esophageal cancer. Second, 2D-PAGE/mass spectrometry-based proteomic analysis of both tumor types suggested that annexin I protein is absent in tumor cells compared with matched normal epithelium from the same patients (8), correlating protein levels with the data generated from allelic DNA changes in esophageal cancer. Lastly, cDNA expression microarray experiments indicated that annexin I mRNA levels were significantly reduced in prostate cancer (9), again correlating protein reduction seen by 2D-PAGE analysis with transcriptional changes in mRNA levels. These three studies indicated that annexin expression may be deranged at a variety of levels in both prostate and esophageal carcinomas. Because of this, annexin I was selected for follow-up analysis from among the many hundreds of macromolecules of interest that were generated in these earlier studies. Annexin I protein levels were evaluated in a study set of invasive prostate and esophageal tumors along with patient-matched normal epithelium and premalignant lesions from both human prostate and esophagus tissue specimens using both immunoblots from LCM-acquired cell populations and conventional IHC analysis.

Materials and Methods

Tissues Specimens. The esophageal specimens studied were from patients who presented to the Shanxi Cancer Hospital in Taiyuan, Shanxi Province, People's Republic of China, and were diagnosed with esophageal cancer. Esophageal sections were snap-frozen immediately after surgery and stored at -70°C until use. The study was approved by the Institutional Review Board of the Shanxi Cancer Hospital and by the United States National Cancer Institute.

Radical prostatectomy specimens were from men with clinically localized prostate cancer. After surgical resection, the specimens were fixed in 70% ethanol and completely embedded in paraffin. Whole-mounted sections were used for IHC and microdissection. All samples were studied anonymously.

LCM and Immunoblotting. Tissue microdissection was carried out after careful pathological examination by a board-certified pathologist (J. W. G.) as described previously using a Pixcell 200 LCM System (Arcturus Engineering,

² LCM, laser capture microdissection; IHC, immunohistochemistry; ABC, avidin-biotin complex method; mAb, monoclonal antibody; RFU, relative fluorescent unit; PSA, prostatic specific antigen; PIN, prostatic intraepithelial neoplasia.

Mountain View, CA; Refs. 8–12). Between 1,500 to 5,000 laser shots (~7,500–20,000 cells) were collected for subsequent Western blot analyses.

Microdissected cells were lysed in 20 μ l of a lysing solution containing a 1:1 mixture of SDS electrophoresis sample buffer [125 mM Tris (pH 6.8), 4% SDS, 10% glycerol, 2% β -mercaptoethanol] and Tissue Protein Extraction Reagent (Pierce, Rockford, IL) directly on the LCM cap. The cell lysate was subjected to SDS-PAGE at 25 mA in running buffer (50 mM Tris-HCl, 380 mM glycine, 4 mM SDS) on a 4–20% gradient acrylamide gel (Novex, San Diego, CA).

Immunoblotting was performed for 2 h using a Bio-Rad Semi-dry blotting apparatus with Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA) as the capture membrane at a constant voltage of 25 V and 10 A/10 cm²/membrane. Protein loads were normalized by blotting membranes against α -tubulin and/or staining membranes with SYPRO Ruby Red protein blot stain (Molecular Probes, Leiden, the Netherlands) according to the recommendations of the manufacturer. Membranes were blocked with Super-Block (Pierce, Rockford, IL) overnight and incubated with the primary antibody at a 1:5,000 dilution (for both the polyclonal and monoclonal anti-annexin I antibodies) in blocking buffer for 2 h under constant rocking. Membranes were then washed with 1 \times Tris-buffered saline four times for 5 min and incubated with a secondary biotinylated antibody at a concentration of 1:2,000 for IgG antimouse (Vector, Laboratories, Burlingame, CA) and 1:35,000 antirabbit (Vector, Laboratories, Burlingame, CA) under constant rocking for 45 min. The membrane was subsequently washed three times for 5 min each in 1 \times Tris-buffered saline and incubated with an enhanced chemiluminescence (ECL-PLUS) substrate (Amersham, Buckinghamshire, United Kingdom). Additional amplification of chemiluminescence signal was performed using the ABC kit from Vector Laboratories according to the manufacturer's recommendations. Blots were exposed to Kodak Bio-Max film for 2–15 min until bands were clearly visible. For Sypro Ruby Red blot staining, blots were subsequently scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantified using IMAGEQUANT (Molecular Dynamics, Sunnyvale, CA).

IHC. Immunohistochemical studies were carried out using ABC staining technique as described previously (13). Briefly, slides were pretreated with 0.3% H₂O₂ in methanol and 10% normal horse serum for 30 min and incubated with either anti-annexin I mAb (1:100 dilution; Transduction Laboratories, Lexington, KY), or anti-PSA mAb (1:100; Scripps, San Diego, CA) for 60 min at room temperature, followed by 1% biotinylated antimouse mAb and the ABC (Vector Laboratories, Burlingame, CA) solution. Development of slides was performed using 0.02% 3',3'-diaminobenzidine solution, followed by counterstaining with hematoxylin, dehydration in ethanol, and clearing with xylene.

Results

Loss of Annexin I Protein in Esophageal Cancer. Immunoblot analysis of annexin I protein expression in patient-matched normal and tumor epithelium from 10 different patients was performed using a commercially available mAb against annexin I. Complete or substantial loss of 38,500 M_r protein was observed in all 10 tumors examined (Fig. 1A).

To demonstrate that the annexin I protein observed on the immunoblots was in fact derived from normal epithelium and was not attributable to "contaminating" stromal cells that were inadvertently procured during LCM, a direct comparison of annexin expression was performed using patient-matched normal epithelium, tumor cells, and stroma. The results in Fig. 1B show that the stromal cells do not contain significant amounts of the 38,500- M_r annexin I protein, thus confirming the presence of the protein in normal epithelium. Two different commercially available antibodies, one polyclonal and one monoclonal (used in Fig. 1A) were used to ensure reproducibility and specificity of the observation. Furthermore, to ensure that the bands that were detected with the anti-annexin antibodies were specific, replicate lysates were loaded and probed with either normal rabbit serum or isotype-matched mouse antibody (Fig. 1B). The results show

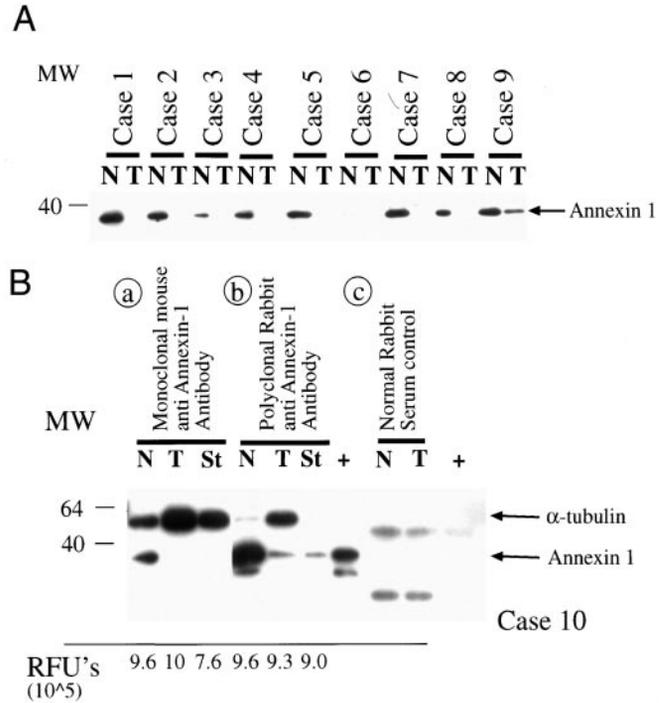


Fig. 1. Ubiquitous loss of annexin-I expression in human esophageal cancer epithelium. A, 2500 cells of patient-matched normal (N) and tumor (T) esophageal cells from nine separate frozen human tissue specimens were microdissected, lysed, and separated on a 4–20% NOVEX SDS-PAGE gel and Western blotted using a monoclonal anti-annexin I antibody (1:1000 dilution). MW, molecular weight (in thousands). B, 6500 microdissected cells of patient-matched normal (N), tumor (T), and stromal (St) cell populations from case 1 were blotted against annexin-I using a mAb (a), rabbit polyclonal antibody (b), or normal rabbit serum (c). A lysate from human endothelial cells, provided by the antibody supplier as a positive control, was also analyzed on the same gel. The blot was probed with an anti-tubulin antibody (1:1000 dilution) as an additional control for loading normalization. Independent verification of normalization load was shown by incubating blots before immunoblotting with SYPRO Ruby Red protein blot stain, measuring relative fluorescent RFUs of each Lane, and quantifying and comparing each Lane by ImageQuant (results shown in RFUs $\times 10^5$). MW, molecular weight (in thousands).

that the protein detected with anti-annexin antibodies is specific and non-cross-reactive.

In all of the immunoblot experiments, protein load was normalized by reblotting the same membranes using α -tubulin as a housekeeping protein, and/or quantifying total protein yield using Sypro Ruby Red protein stain. For example, the results for total protein load assessment using Sypro Ruby Red blot stain are shown in Fig. 1B (expressed in RFUs) for each Lane.

Loss of Annexin I Protein in Early Stages of Tumorigenesis. To determine whether the loss in annexin 1 expression occurred early in the development of tumorigenesis, lysates from patient-matched (case 11) microdissected normal epithelium, high-grade dysplasia, and frankly invasive carcinoma were also analyzed for annexin 1 expression. Fig. 2A shows that the dysplastic cells from a patient-matched microdissected study set express significantly lower levels of annexin I than the corresponding normal epithelium. Interestingly, the premalignant cell population expresses a 52,000- M_r protein which specifically cross-reacts with both the polyclonal and monoclonal anti-annexin 1 antibodies (data for polyclonal antibody shown) that is not observed in the lysates from either the normal or the tumor epithelium from the same patient. We extended this observation to more patient samples using LCM to procure a more detailed longitudinal patient-matched epithelial study set for annexin 1 expression analysis (Fig. 2B). The results show that whereas annexin 1 expression is dramatically reduced in all invasive epithelial cells, early loss can occur at either the junction between high-grade dysplasia and invasive phenotypes or at the low grade-to-high grade transition.

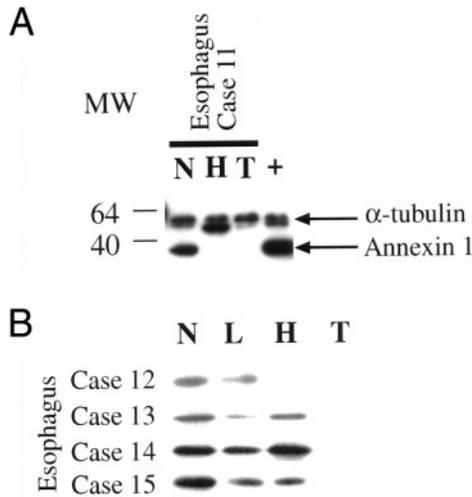


Fig. 2. Loss of annexin I expression in premalignant esophageal epithelium. *A*, 4,500 microdissected epithelial cells of human esophageal, patient-matched normal (*N*), high-grade dysplasia premalignant (*P*), and tumor (*T*) cell populations from an ethanol-fixed tissue specimen were subjected to annexin I Western blot analysis using a polyclonal rabbit anti-annexin I antibody (1:1000 dilution). Normalization of protein load was analyzed and confirmed using an anti- α -tubulin antibody (expected size of 54,000 M_r). MW, molecular weight (in thousands). *B*, 4,500 microdissected epithelial cells of human esophageal, patient-matched normal (*N*), low-grade dysplasia (*L*), high-grade dysplasia (*H*), and tumor (*T*) cell populations using ethanol-fixed tissue specimens from four patients were blotted against annexin-I using a polyclonal rabbit anti-annexin I antibody (1:1000 dilution). Normalization of protein load was analyzed and confirmed using an anti- α -tubulin antibody (data not shown).

Loss of Annexin I Expression in Human Prostate Cancer. To investigate the protein expression status of annexin I in prostate cancer epithelium, which was seen to have reduced annexin mRNA levels, patient-matched tumor and normal epithelial cell populations were procured by LCM from one case and annexin I levels analyzed by Western blot. For comparison, microdissected tumor and normal epithelium from an esophageal cancer case studied previously (case 7) was run adjacent to the prostate microdissections (Fig. 3*A*). Normalization for protein loading was confirmed by reprobing the blot with both anti-PSA and anti- α -tubulin antibodies. In an identical manner to the esophageal expression patterns, the prostate tumor cell population expressed a dramatically reduced level of annexin I protein as compared with the corresponding normal epithelium. Furthermore, two other prostate tissue specimens were analyzed for annexin I expression, and both showed a dramatic reduction in annexin I expression in the tumor epithelium cell populations (Fig. 3*B*).

IHC Analysis of Annexin I Expression. To confirm the observations seen from the Western blot analysis of esophageal and prostate cancers and to determine annexin I intracellular localization, IHC analysis was performed on a study set of 14 prostatectomy specimens (4 ethanol-fixed, paraffin embedded, whole-mounted, and 10 frozen sections) and 5 separate esophageal specimens. A representation of the anti-annexin I IHC for the normal, premalignant, and invasive carcinoma from one of each of these tissue cases is shown. As seen in Fig. 4, *A* and *B*, the polyclonal anti-annexin I antibody showed strong annexin I staining in the esophageal normal epithelium (*panel A, left side*) with reduced annexin I expression in the premalignant high grade dysplastic epithelium (*panel A, right side*) and absent annexin I expression in the invasive esophageal carcinoma (*panel B*). This pattern of annexin I staining reactivity was identically observed in the other four esophageal cases (data not shown). Adjacent recut sections were stained with an anti-pan cytokeratin mAb and showed consistent and strong staining in normal, dysplastic, and malignant glands (data not shown). Similarly, annexin I IHC analysis of an ethanol-fixed prostate cancer tissue specimen revealed strong staining of normal

epithelium with dramatically reduced staining for the malignant glands (Fig. 4*C*). Staining was absent or dramatically reduced in the epithelial layer of high-grade PIN glands, with retention of strong annexin I staining in the basal layer (Fig. 4*D*). This pattern of annexin I staining reactivity was identically observed in the other 13 cases regardless of the fixation condition used (data not shown). Adjacent recut sections were stained with an anti-PSA mAb and showed consistent and strong PSA staining in normal, high-grade PIN, and malignant glands (data not shown).

Discussion

Research efforts that use a molecular profiling approach toward identification of alterations in human tumors are becoming increasingly important. A positive and negative aspect of these types of studies is that they generate vast data sets for analysis and produce large numbers of genes and proteins that may potentially be important in tumor formation or progression. Thus, it is critical to devise strategies that permit rational prioritization of a manageable number of genes for follow-up analysis. Important criteria for investigators to consider include: (*a*) the frequency of the alteration in tumors; (*b*) the stage of tumor progression in which the alteration occurs, and (*c*) the number of different tumor types in which the gene (or pathway) of interest is dysregulated. Three separate and independent molecular profiling studies in our group identified annexin I previously as potentially important in two different tumor types: esophageal and prostate cancer. Each of these independent studies indicated a potential for annexin I protein expression level changes in the tumor epithelium. Therefore, we prioritized annexin I for in-depth analysis. The results of the present study confirm our previous observations, but in a more comprehensive and larger set of patients, and importantly, indicate that the loss of annexin I protein is an early event that occurs in premalignant lesions in both tumor types. Thus, the high frequency of dysregulation in tumors (25/25 esophageal cases and 17/17 prostate cases; $P \leq 0.05$), the early stage at which the protein is lost, and the

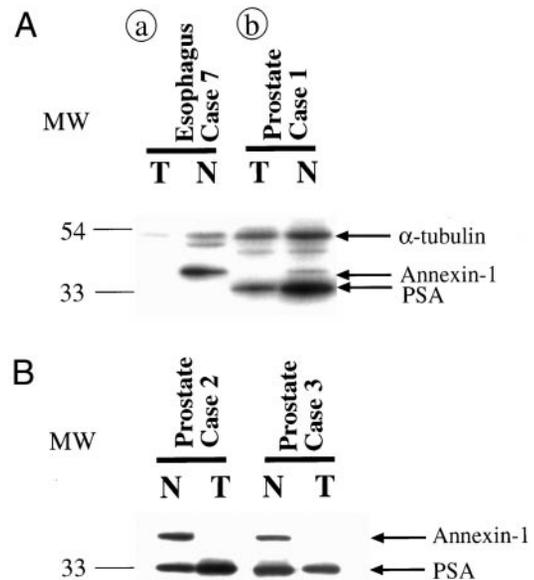


Fig. 3. Decreased annexin I expression in human prostate cancer epithelium. *A*, 6,000 normal (*N*) and tumor (*T*) esophageal epithelial cells and 25,000 normal (*N*) and tumor (*T*) prostate epithelial cells from frozen human tissue specimens were microdissected and subjected to immunoblot analysis against annexin-I. Normalization of proteins between these two tissue types was established by probing against α -tubulin (expected size of 54,000 M_r), and PSA (expected size of 33,000 M_r). MW, molecular weight (in thousands). *B*, 25,000 normal (*N*) and tumor (*T*) prostate epithelial cells from two other human cancer tissue specimens were analyzed using both anti-annexin I and anti-PSA antibodies as in *A*. MW, molecular weight (in thousands).

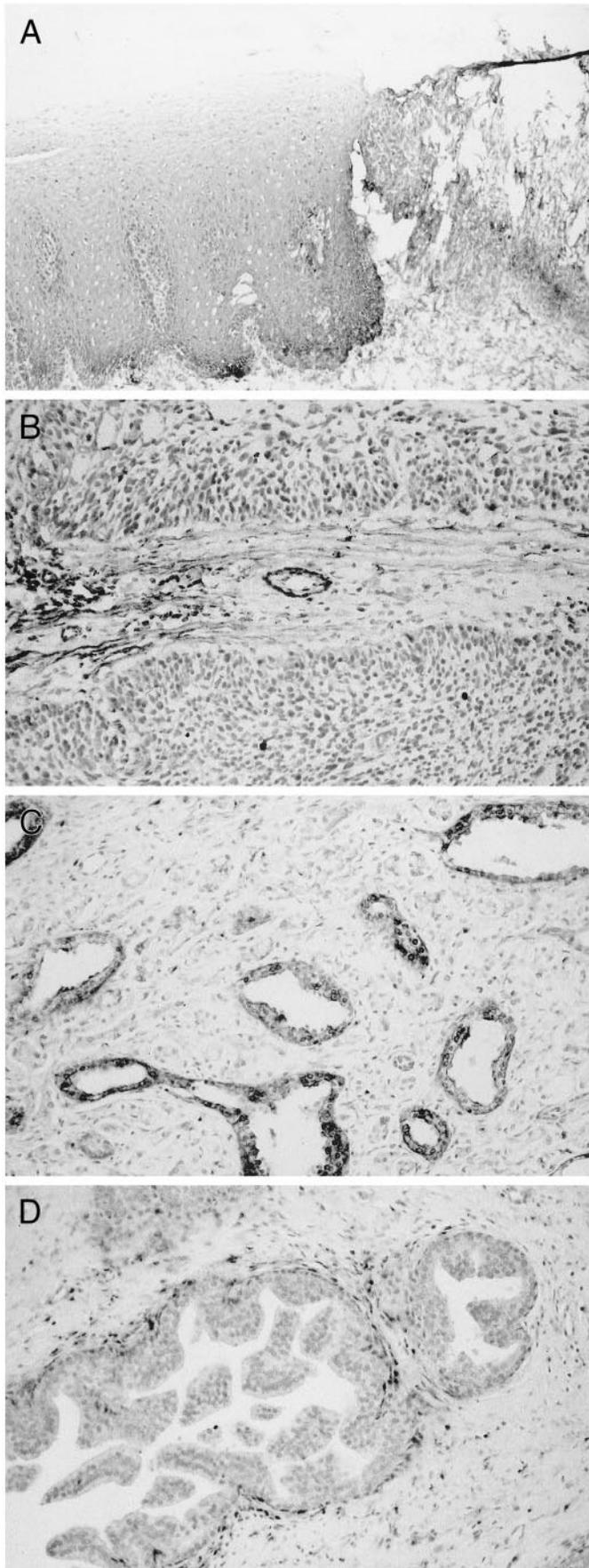


Fig. 4. Immunohistochemical analysis of annexin I expression in human prostate and esophageal cancer tissue specimens. A, a photomicrograph of esophageal epithelium from ethanol fixed, paraffin embedded human tissue specimens. On the left side is normal

fact that annexin I is altered in tumors of diverse cellular lineage (squamous carcinoma, adenocarcinoma) all suggest that annexin I may be fundamentally important in human tumorigenesis.

Furthermore, we confirmed these results using two different commercially available antibodies to annexin I. Interpretations of protein expression based on IHC alone need to be examined cautiously, as findings can often be misleading, not reproducible, and highly subjective. Moreover, positive staining may not provide information about some of the important posttranscriptional or posttranslational alterations in proteins that affect its mobility and can be detected by SDS-PAGE separations. Western analysis was critical in determining both protein size calculations and relative levels of protein abundance. For example, because we used a polyclonal antibody that recognizes epitopes on the entire annexin I protein, we could conclude that alterations in annexin I expression did not arise from proteolytic clipping because no low molecular weight bands were observed on Western blot. LCM provided a means to procure patient-matched normal, premalignant, and tumor material for our discovery-based genomic and proteomic efforts. The use of this technology was crucial to our findings, especially in the case of proteins such as annexin I, where expression was found to be lost as a cause or consequence of the tumorigenesis.

Annexin I (lipocortin I) is a pleiotropic, calcium-dependent phospholipid binding protein (14). Ascribed functions include, among many, inhibition of phospholipase A2 (15) and mediation of apoptosis (16). Annexin I has also been shown to be a substrate for epidermal growth factor receptor (17). Previous reports have suggested that annexin I protein is actually overexpressed in some malignancies including breast cancer (18). However, in other studies, loss of inhibition of annexin I appears associated with a lack of cellular differentiation (19, 20). Likewise, our findings show that annexin I protein expression is decreased in human esophageal and prostate cancer. Comparative analysis of a microdissected human breast cancer tumor lysate with both prostatic and esophageal tumor and normal cells indicated that the annexin I expression in malignant breast epithelium is significantly higher than in the esophageal and prostate tumor cells (data not shown). The etiology of reduced annexin I protein expression is not known. Possible mechanisms include genomic deletions, truncating mutations of the *annexin I* gene, hypermethylation of the promoter with subsequent loss of transcription, or alterations in post-translational processing of the protein. Defects of intracellular transport or protein storage that lead to reduced intracellular levels of annexin I may also be responsible. Follow-up studies to determine the mechanism of annexin I protein loss in each tumor type are currently underway.

Unlike past efforts to analyze annexin expression in human tumors, we have used LCM-based Western analysis of patient-matched longitudinal cell populations with IHC as a means to more comprehensively validate our findings. These longitudinal study sets included both low-grade and high-grade premalignant lesions so that, for the first time, direct comparisons between these important cell populations could be analyzed for patterns of protein expression relative to their normal and frankly malignant epithelial counterparts. In the future, the use of these LCM-procured longitudinal cell sets could

esophageal epithelium, showing strong staining by polyclonal antibodies to annexin I. On the right side is dysplastic esophageal epithelium, with reduced annexin I expression. B, a high-power photomicrograph of invasive esophageal carcinoma, without immunoreactivity to annexin I. The normal endothelium of a vessel stains positive. C, a high-power photomicrograph of the prostate from ethanol-fixed paraffin-embedded human tissue specimens. The benign prostatic epithelium stains positive for annexin I, whereas the surrounding malignant glands are negative. D, a high power photomicrograph of high grade PIN showing strong annexin I staining of the basal layer of the glands and reduced expression in the epithelial layer.

become an important tool for the molecular characterization of cancer and disease-related proteins. The ability to identify clinically important therapeutic targets or biomarkers for early detection of cancer will ultimately rely on the ubiquity with which the protein of interest changes with respect to large population cohorts. We feel that the proteins with the best chances of clinical utility will be discovered through these longitudinal patient-matched disease progression study sets. Those proteins whose expression patterns consistently change not only between different patients, but also within the patient-matched sets will most likely reflect the most important candidates for additional investigation in large validation studies.

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