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

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Abstract

Annexin 1 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 1 protein expression compared with patient-matched normal epithelium (P ≤ 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 1 may be an essential component for maintenance of the normal epithelial phenotype. Additional studies investigating the mechanism(s) and functional consequences of annexin 1 protein loss in tumor cells are warranted.

Introduction

The development of rational approaches to the diagnosis and treatment of cancer is dependent upon 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 disregulated 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 even 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).

In the present study, we evaluated the expression of annexin 1 protein in two disparate tumor types: squamous cell carcinoma of the esophagus and prostate adenocarcinoma. Annexin 1 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 chromosomes arm 9q near the annexin 1 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 1 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 microarray experiments indicated that annexin 1 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 1 was selected for follow-up analysis from among the many hundreds of macromolecules of interest that were generated in these earlier studies. Annexin 1 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 esophageal 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, California).
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 M₀ 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-M₀ 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 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 I 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 I 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-M₀ protein which specifically cross-reacts with both the polyclonal and monoclonal anti-annexin I 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 I expression analysis (Fig. 2B). The results show that whereas annexin I expression is dramatically reduced in all invasive epithelial cells, early loss can occur at the junction between high-grade dysplasia and invasive phenotypes or at the low grade-to-high grade transition.
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. 3A). Normalization for protein loading was confirmed by reprobing the blot with a-\(\alpha\)-tubulin antibody (data not shown).

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. 2, A and B, the polyclonal anti-annexin I antibody showed strong a-\(\alpha\)-tubulin staining 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 disregulated. 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 disregulation 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
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 pleotrophic, 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 posttranslational 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

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 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.

References

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