Journal of Cellular Biochemistry 104:1988–1993 (2008)

Nuclear Microenvironments in Cancer Series

Nuclear Structure as a Source of Cancer Specific Biomarkers

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Abstract There are few biomarkers that have been developed which have proven clinical utility for the detection and prognosis of cancer. Cancer is diagnosed today, in large part, by examining cells under the microscope and determining the shape and texture of the nucleus. The molecular underpinnings of this hallmark of cancer are the components of the nuclear matrix. Utilizing proteomics focused on this subset of proteins, biomarkers have been identified that are specific for cancer types including prostate, colon and bladder cancer. These cancer biomarkers now serve as the basis of assays which can specifically identify individuals with cancer by sampling their blood and/or urine. In addition, these may serve as potential therapeutic targeting or imaging approaches. J. Cell. Biochem. 104: 1988–1993, 2008. © 2007 Wiley-Liss, Inc.

Key words: nuclear matrix; nuclear structure; tumor markers; biomarkers

CLINICAL SIGNIFICANCE

With the advent of numerous molecular technologies, many advances have been made in our understanding of cancer. Despite these advances, few of these, if any, have translated from the laboratory to being used as biomarkers to detect cancer to serve as prognostic indicators in blood or urine-based assays. It remains apparent that the earlier we can detect cancers and treat them, the more successful we will be in curing them. Much of the prevention strategies rely upon the identification of high-risk individuals or those that may have very early disease. In addition, for a number of cancers, including prostate cancer, it is often difficult to differentiate the cancers which are more aggressive from those that may not cause individuals life threatening problems within their lifetimes. A number of the current biomarkers that are used have been around for many years. These include prostate specific antigen (PSA) [Catalona et al., 1997; Balk et al., 2003; Thompson et al., 2005] and carcinoembryonic antigen (CEA). These commonly used tests suffer from their lack of cancer specificity, and these proteins are often found to be elevated within the bloodstream of individuals with non-cancerous conditions. To address this urgent need, many approaches have focused on the characterization of the proteome that exists within body fluids, including blood and urine. These approaches have been difficult in that there are a number of abundant and interfering proteins which need to be subtracted out in order to find biomarkers that have the potential to be reasonably specific for the disease. Gene expression analyses and proteomic analyses of tumor tissues in comparison to normal tissues have, indeed, identified many differences, but the translation of these

Robert Getzenberg holds a patent for some of the technologies described in this manuscript. Several of these patents are owned by the University of Pittsburgh and Johns Hopkins University, and have been licensed to Onconome Inc. He has also received a research grant from Onconome Inc, and is a consultant to the company. The terms of this arrangement are managed by the Johns Hopkins University in accordance with its conflict of interest policies. Dr. Leman does not declare a conflict of interest.

Grant sponsors: National Institutes of Health, National Cancer Institute; Grant numbers: CA65463 and CA084968 and a research grant from Onconome.

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Received 9 March 2007; Accepted 12 March 2007

DOI 10.1002/jcb.21363

into biomarkers that can easily and reliably be detected by noninvasive techniques has been difficult.

Our goal, when developing new cancer biomarkers, was to take a step back and begin to understand some of the fundamental properties of a cancer cell. The most obvious, consistent property of a cancer cell is that a pathologist can look under a microscope and identify such a cell and differentiate it from a normal one. In fact, this is, today, our definition of cancer; that is, the pathologists can tell us whether the sample that they are examining contains cancers cells or not. Our assumption was that there must be something at the molecular level that is the correlate of what the pathologist is seeing under the microscope. One of the hallmarks of the pathologic changes that the pathologist observes is alterations in the shape and texture of the nucleus [Konety and Getzenberg, 1999]. These nuclear modifications are found in all cancer cells and represent cancer specific signatures. With these differences in the cancer nucleus being such fundamental aspects of the cancer process, our hypothesis was that the molecular foundation of these nuclear changes might serve as biomarkers of the disease. The underpinnings of nuclear shape and texture are the structure of the nucleus, the nuclear matrix. This nuclear matrix, which was originally discovered in Berezney and Coffey [1974] is the dynamic scaffolding structure of the nucleus which organizes all of the nuclear processes, including DNA replication, transcription, message translocation, splicing, etc. The functions of the nuclear matrix are beyond the discussion in this review, but this nuclear structure serves as the organizing element of the nucleus, and, therefore, changes in the nuclear matrix may not only reflect alterations in nuclear shape but also in the fidelity by which processes within the nucleus are carried out [Konety and Getzenberg, 1999]. As a scaffolding of a building can predict the shape of the building, this structure of the nucleus, we believe, also predicts the shape of the nucleus. Utilizing an approach that we termed, "focused proteomics," we carried out proteomic analysis of nuclear matrix proteins from cancer cells in comparison to their normal counterparts. This approach has a number of advantages over some of the more general proteomic and gene expression approaches. First, this is focusing on a specific set of proteins—the nuclear matrix proteins—that represent, what we believe to be, some of the fundamental changes which occur within a cancer cell. Secondly, these are proteins with low abundance in that the nuclear matrix, in total, represents less than 1% of the total protein composition of the cell and approximately 10% of the nuclear proteins. These low abundant proteins will, therefore, have a minimal potential to be identified through some of the more general approaches in that these proteins may, indeed, often be missed. Furthermore, the relatively insoluble nature of many of these proteins makes them difficult to separate through common approaches. By focusing on the nuclear matrix, we were also able to eliminate some of the highly abundant and interfering proteins which often exist in resolving complex protein mixtures; for example, those of the blood and/or tissue. Finally, as we described above, this is a hypothesis driven approach where we are focusing on proteins that we believe are fundamental in the cancer process. We will describe below how this approach has been utilized to identify and characterize a series of cancer specific biomarkers for a number of cancer types. These examples will not be comprehensive in nature but will show the potential to utilize the nuclear matrix as a source for biomarkers as well as perhaps therapeutic targets which may be specific for the cancer process.

PROSTATE CANCER

Prostate cancer is the leading cancer diagnosed in men in the United States and represents the second major cause of cancer deaths within this group [Jemal et al., 2006]. It is a worldwide problem, and as the population ages this problem is only increasing. The only currently available blood-based biomarker for prostate cancer (PSA) has been in use for more than 25 years. While this biomarker has changed the course of the disease in that few men today present with metastatic disease as initial presentation, it has also resulted in a large number of men having to undergo biopsies of their prostates with only a small percentage actually having the disease. There clearly is a need for markers that are specific for prostate cancer as well as those that can differentiate the prostate cancers that have the ability to progress and kill the patient from the prostate cancers in which the patient might die from

other causes. Utilizing an approach that we summarized above, we examined the nuclear matrix protein composition of prostate cancer tumors in comparison to normal prostates. In general, we isolate nuclear matrix proteins from prostate cancer samples and normal control samples. We then separate them using high-resolution, two-dimensional electrophoresis. Protein spots that are consistently found to be associated with the cancer or normal tissues are then sequenced by mass spectrometry or other approaches [Getzenberg et al., 1991; Partin et al., 1993]. In doing so, we have identified a number of proteins that appear to be only found within the nuclear matrix of prostate cancer cells that are not found within normal prostatic cells [Getzenberg et al., 1991; Partin et al., 1993]. Two of these proteins we have termed early prostate cancer antigen (EPCA) and EPCA-2 along with some more recent identifications, including C-21. EPCA was the first of these biomarkers to be characterized. The expression of this marker was intriguing in that it was found not only within the prostate cancer itself but was also found within normal, adjacent areas of the prostate in men with the disease [Dhir et al., 2004]. EPCA expression was found to be absent in men without prostate cancer as well as in those with BPH [Dhir et al., 2004]. Their findings have been substantiated by an independent group [Uetsuki et al., 2005]. One of the potential clinical applications of this marker is to utilize it in men being biopsied for prostate cancer to determine, even in a biopsy which appears to be normal, if, indeed, the presence of EPCA can predict the presence of prostate cancer. Initial experiments evaluating the immunohistochemical staining of prostatic biopsies utilizing an antibody raised against this protein have been quite promising. The application of this approach, in a pathologic setting, is currently underway. We are hopeful that EPCA can help discriminate between men who have prostate cancer despite the fact that their biopsies are negative, but perhaps even more importantly be able to reassure men that despite the fact that their PSA levels may be elevated that they do, indeed, not have prostate cancer within their prostates. Further validation and additional analysis of this biomarker are certainly needed. In addition to the tissue-based detection of EPCA, we have been successful at detecting EPCA in the plasma of men with prostate cancer.

EPCA-2 was the second of our biomarkers to be pursued. This protein was different than EPCA in that it was not found throughout the prostate of men with prostate cancer but had a more characteristic pattern of being found in the tumor but missing in the normal, adjacent areas as well as in the normal areas of men without the disease. Antibodies raised against this protein have been utilized to develop a test which can be applied to the blood and which, indeed, detects this protein within the blood of men with prostate cancer. This simple blood test has now been applied to more than 385 serum samples to evaluate its characteristics in identifying men with prostate cancer. In a recent study, in which we have evaluated men with prostate cancer from those without the disease, including men with BPH as well as individuals with other types of benign conditions and cancer types, EPCA-2 has been shown to be highly specific for prostate cancer. The specificity for this marker was shown to be 97% in these studies with a sensitivity of defeating prostate cancer of 94% [Leman et al., 2007a]. With some recent optimization to the assay, we have been able to demonstrate that EPCA-2 levels are extremely low or non-detectable in females, and in men undergoing prostatectomies, the levels go from being elevated to basically undetectable [Leman et al., 2007a]. EPCA-2 has also been shown to be able to differentiate between men with disease that is contained within the prostate (organ confined disease) from disease that has already spread outside the prostate at the time of surgery (non-organ confined disease) [Leman et al., 2007a]. This distinction is an important one, and, while it does not definitively show that EPCA-2 can identify the aggressive prostate cancers from the less aggressive ones, it does, indeed, reflect promise in the ability of this biomarker to make such a separation. With these intriguing results, a number of additional studies are currently underway to validate this marker as well as to fully characterize its potential clinical use. At the same time, we are intrigued to understand the biological function of this protein in the cancer process.

We are currently also in the process of characterizing several other prostate cancer associated nuclear matrix proteins. While further validation is clearly needed, the proteins that have been identified to date appear to be exciting possibilities that may change the clinical paradigm of the disease.

COLON CANCER

The early detection of colon cancer has, indeed, been shown to have significant clinical impact [Hardcastle et al., 1996; Mandel et al., 1999; Bond, 2000]. In fact, the use of colonoscopy as a screening tool for the disease is promoted in aging individuals. Despite the efficacy of colonoscopy, it remains utilized by only a small percentage of the population, and few have repeat colonoscopies as recommended. A simple blood test that would provide an opportunity to identify individuals with colon cancer or perhaps even to identify those at higher risk for the disease that may require colonoscopies would be of great value. Utilizing a similar approach described above for prostate cancer, we have been working on identifying colon cancer associated nuclear matrix proteins which we can develop into biomarkers for the disease. Utilizing our focused proteomic approach, we have identified a number of nuclear matrix proteins which appear to be associated with colon cancer [Brunagel et al., 2002a,b, 2003]. Among these protein changes are two biomarkers of interest—colon cancer specific antigen-3 (CCSA-3) and CCSA-4. Utilizing antibodies that we have recently produced against these proteins, we have developed assays that can detect these proteins within the blood. In these studies, we have examined the serum of individuals undergoing colonoscopy to determine the ability of both CCSA-3 and CCSA-4 to identify those with colon cancer [Leman et al., 2007b]. In the study populations, individuals were shown upon colonoscopy to either be normal, to have hyperplastic lesions, or to contain adenomas, either advanced or non-advanced. In addition, there was a subset of patients that were shown to have colon cancer. Blood levels of both CCSA-3 and CCSA-4 were found to be significantly elevated in the individuals with colon cancer. In addition, a number of those with advanced adenomas also had elevated levels of these proteins. CCSA-3 and CCSA-4 are both quite specific for colon cancer and are not found to be present in other cancer types or benign diseases [Leman et al., 2007b]. Validation studies are currently being performed for these assays utilizing reference sample sets. The potential for these assays to determine if, indeed, an individual may have colon cancer or at the minimum an advanced adenoma and, therefore, significantly increased risk for colon cancer is quite promising. This blood test might be able to be utilized to provide the identification of individuals who require a colonoscopy with the hope that these individuals can be identified at an early and more curable stage.

BLADDER CANCER

One of the first cancer types that we examined was bladder cancer. Bladder cancer is a unique model in that most tumors are initially resected inside the bladder, and, therefore, temporal changes can be studied within the organ. In addition, bladder cancer is relatively unique in that the bladder itself is bathed within a body fluid that is easy to sample, i.e., urine. Utilizing the high resolution, two-dimensional approach that we have performed to examine nuclear matrix proteins, we identified a number of proteins that were specific for bladder cancer that were not identified in other cancer or normal tissue types. These proteins were termed the BLCA proteins (1–6) [Getzenberg et al., 1996]. Most of the work has been done with BLCA-4. Utilizing sequences that we obtained from the protein, the gene encoding BLCA-4 was deciphered. It was determined that BLCA4 appears to be a member of the ETS transcription factor family [Van Le et al., 2004]. This family has come under renewed interest with the recent fusion genes that have been identified by the Chinnaiyan group at the University of Michigan [Tomlins et al., 2005]. BLCA-4 appears to be closest in the ETS family to ELK3 [Van Le et al., 2004]. Despite its association with ELK3, it appears to be distinct from this protein, and it has several regions which do not match the sequence of this protein. Utilizing antibodies that we have produced against BLCA-4, we were able to develop an assay which could detect the protein within the urine of patients with bladder cancer. Utilizing this urine assay, we are able to discriminate between individuals with bladder cancer and normal individuals as well as those with other benign diseases which often complicate the analysis of urine samples [Konety et al., 2000a,b; Van Le et al., 2005]. These benign diseases include cystitis, overactive bladders and BPH. The specific discrimination of those with bladder cancer provides the potential that BLCA-4 may have a high clinical impact. Current studies are underway to both determine the clinical significance of the BLCA-4 urine assay as well as

the functional role of this protein within the cancer process.

At the functional level, we now know that by overexpressing this protein in bladder cells, we are able to impart a significant growth advantage in these systems. We have also identified a number of proteins which are both up and down regulated by overexpressing BLCA-4 which have been shown to be involved in the cancer process particularly that involved with more advanced cancers [Myers-Irvin et al., 2005b]. It, therefore, seems that BLCA-4 may play a role in helping to regulate the cancer process, perhaps through ritual and regulating a number of genes which may be important in the development of advanced disease. At the clinical level, the urine assay that we have produced against BLCA-4 continues to show good separation between those with bladder cancer and those without the disease. Additional validation studies are currently underway. In addition, we are examining several high-risk groups for bladder cancer, including those with occupational exposures as well as those with spinal cord injuries which are known to be at a much higher risk of bladder cancer than the general population. Another of our bladder cancer associated nuclear matrix proteins is BLCA-1. This protein was shown to also be associated with bladder cancer, and an assay was produced which could detect it within the urine of individuals with bladder cancer. Although this marker is not as specific for bladder cancer as BLCA-4, it does appear to be a useful tool in the identification of individuals with the disease [Myers-Irvin et al., 2005a]. We also have several proteins associated with normal bladder that are missing in bladder cancer (BLNL 1–3). We have proposed that these proteins may be involved in the suppression of cancer and are turned off during the development of the disease. At this point, few studies have been performed on these proteins to further understand their properties.

One of the currently used urine-based biomarkers for bladder cancer, NMP-22, is actually a nuclear matrix protein. This protein NuMa is associated with the nuclear mitotic apparatus and is a component of the nuclear matrix. This assay is currently FDA approved for use in bladder cancer, and although it is not a specific change associated with bladder cancer, it demonstrates the clinical utility of the nuclear matrix as a diagnostic target.

OTHER CANCER TYPES

We have performed the types of analyses described above for prostate, colon and bladder cancer for other cancer types. Among these cancer types was renal cell carcinoma. The RCC associated nuclear matrix changes appear to be quite exciting, and we are in the process of developing serum based detection tools for these biomarkers [Konety et al., 1998; Cannon and Getzenberg, 2006; Cannon et al., 2007]. Other groups have studied nuclear matrix protein changes that are associated with breast cancer as well as ovarian cancer.

CONCLUSIONS

As described in the examples above, the initial concept of utilizing changes in nuclear structure as cancer specific biomarkers has been substantiated. For a number of cancer types, clinically useful assays have been produced which focus on these nuclear structure changes. These nuclear structure changes provide specific alterations associated with cancer that may be the molecular correlates of what the pathologist is observing under the microscope. In fact, these may represent some of the few documented changes which are specifically associated with cancer. In addition, these changes may be playing an active role in the cancer process by contributing too many of the nuclear changes which are observed in the functionality of a cancer cell. Nuclear matrix changes appear to be a rich source for potential cancer biomarkers, and may, indeed, reveal important cellular clues about the cancer process. These markers may also provide targeting systems for therapeutic agents as well as revealing processes which may be able to be explored for therapeutic means.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute of Health, National Cancer Institute (CA65463 and U01 CA084968) and a research grant from Onconome, Inc.

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