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Nuclear Structural Proteins as Biomarkers of Cancer

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Abstract The regulation of cell processes is integrally connected to cellular and extracellular structure. Studies over the past three decades have demonstrated the complex interactions of cell structure and function. The relationship of cellular structure and function has perhaps been most studied in the transformed cell. The hallmark of transformation is alterations in the shape of the cell and the nucleus. Many of the cellular alterations observed in the cancer process are structural, including changes in extracellular matrix-cytoskeletal interactions, cytoskeletal elements, as well as nuclear structure. This review focuses on the structural components of the nucleus, the nuclear matrix, and their role in the cancer process and the use of these structural components of the nucleus, the nuclear matrix, and their role in the cancer process and the use of these structural components as cancer specific biomarkers. J. Cell. Biochem. Suppls. 32/33:183–191, 1999.

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The presence of intranuclear proteins that contribute to the maintenance of nuclear shape and organization has been theorized for quite some time. A set of proteins that are resistant to extraction by high molar concentrations of salt have been described since the 1940s and referred to as the "residual protein fraction" or "nuclear network" [Pederson, 1998]. Smetana et al. [1963] demonstrated a ribonucleoprotein network in the nucleus of Walker tumor and rat liver cells after extraction of the soluble proteins and other nuclear constituents. The "nuclear matrix" was first described as a structural and functional entity by Berezney and Coffey [1974] in rat liver cells. The residual nuclear matrix is derived after serial extraction of lipids, soluble proteins, intermediate filaments, DNA, and most of the RNA. It is the nonhistone, protein, and RNA based scaffolding of the nucleus. The structure of the nuclear matrix can be visualized as being composed of three parts—the nuclear membrane, which comprises the nuclear lamina and pore proteins, the nucleolar proteins, and the granular nuclear matrix itself, which extends from the nucleolus to the nuclear lamina [Bosman, 1996]. Further analysis of the nuclear microarchitecture has shown that the nuclear skeleton may be composed of core filaments that extend radially from the nucleolus to the nuclear lamina, a "diffuse skeleton" of filaments composed of protein-nucleic acid complexes attached to the core filaments and nuclear bodies, interspersed between the filaments [Hozak, 1996]. The precise composition of the core filaments and the diffuse skeleton of filaments remains to be determined. A large number of proteins have now been shown to be nuclear matrix proteins. These include structural proteins such as F-actin and the lamins; proteins involved in replication, transcription, and splicing complexes, as well as transcriptional regulators. Alterations in nuclear structure including dramatic changes in morphology are a distinguishing characteristic of most cancers. Since the nuclear structure is maintained predominantly by the nuclear matrix, it is logical to assume that alterations in nuclear shape or structure that occur with neoplastic transformation are accompanied by changes in nuclear matrix composition or archi-

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tecture, or both. This article reviews the structure and function of the nuclear matrix and its components and their potential role in the neoplastic process. Recently considerable attention has been directed toward using the nuclear matrix as a diagnostic tool to detect cancers through the identification of various cancer specific nuclear matrix proteins. We also review recent progress in using nuclear matrix proteins as diagnostic/prognostic markers for cancer and attempt to identify critical issues and potential future directions for research in this area.

FUNCTIONAL IMPORTANCE OF THE NUCLEAR MATRIX

The nuclear matrix has been demonstrated to be intimately involved in many nuclear functions. Recent evidence indicates that the nuclear matrix plays a role in DNA organization, replication, RNA synthesis, and RNA splicing and can function as binding sites for steroid hormone receptors and other proteins [Replogle-Schwalb et al., 1996; Nangia et al., 1998]. A number of transcriptional regulators have also been found to be associated with the nuclear matrix [Nordozza et al., 1996; Stein et al., 1998; Steinonen et al., 1998]. Nuclear DNA is configured into "loop domains," which are about 60 kb in length; these loops are attached to the nuclear matrix at their bases. These segments of the DNA loops, which attach to the nuclear matrix, have been termed matrix attachment regions (MARs) or scaffold attachment regions (SARs) [Vogelstein et al., 1980; Luderus et al., 1992]. Several MARs have been shown to be associated with transcriptionally active genes. Some of the proteins associated with MARs include topoisomerase II [Berrios et al., 1985], SATB-1 in thymocytes [Dickenson et al., 1992], the 240-kd NuMA protein [Luderus et al., 1994], and the p114 protein, found in breast tumors [Yanagisawa et al., 1996]. Other nuclear proteins that are part of the nuclear matrix can also bind MARs, such as lamins A and C [Luderus et al., 1992, 1994; Hakes and Berezney, 1991].

PROTEIN CONSTITUENTS OF THE NUCLEAR MATRIX

A whole host of other proteins have also been identified as being constituents of the nuclear matrix. These include autoantigens, fibronectin, keratin-like proteins, oncogene products, transcription factors, primer recognition proteins, enzymes such as DNA and RNA polymerases, phospholipases, and protein kinases [Martelli et al., 1996]. Both the functional and structural implications of these nuclear matrix constituents remain to be elucidated. Nakayasu and Berezney [1989] have identified several polypeptides that are present in cells and tissues of various types, which they called "nuclear matrins." Many of the proteins that can be isolated from the nuclear matrix fraction are functional components of the mitotic spindle in cells [Kallajoki et al., 1991; Wan et al., 1994]. In this context, the nuclear mitotic apparatus protein or NuMA is in found most cells and is one of the proteins that is part of the nuclear matrix. One very abundant nuclear matrix protein B23/numatrin is a 38-kD protein that serves as a shuttle protein in cells and is involved in cytoplasmic-nuclear transport [Martelli et al., 1995]. Topoisomerase II is also known to be a constituent of the nuclear matrix and is abundantly present in the metaphase nucleus. It is known to be involved in the topological organization of DNA [Earnshaw and Heck, 1985]. Although the existence and association of many of these proteins with the nuclear matrix have been demonstrated, their functional significance as components of the nuclear matrix remains to be determined.

MORPHOLOGIC PARAMETERS AND CANCER DIAGNOSIS

Pathologic identification of cancer is based on the presence of certain unique features of tumor cells. Alterations in nuclear shape, size and intranuclear DNA are all cardinal signs of cancer. Several of these changes are discernable on microscopic examination of tumor tissue sections. Some of these parameters can be quantified using image analysis. Some of the quantifiable measures, termed nuclear morphometric descriptors, are nuclear shape, nuclear roundness factor, size, DNA content (ploidy), and DNA distribution [Veltri et al., 1998]. These determinations can be made using sophisticated image analysis systems. Except for overall nuclear morphology, most of these measures are only applicable once cancer has been diagnosed, to discriminate between tumors of varying biologic potential and behavior. These parameters have been used to analyze tumors of various types with varying success rates. Although they may play a significant role in pre-

dicting tumor behavior and prognosis, their role in cancer diagnosis is still somewhat limited by complexity, expense, and relevance. Alterations in basic nuclear matrix structure and composition may underlie the changes in many of these nuclear morphometric parameters. In a similar fashion, many other features of cancer cells, such as chromosomal rearrangements, translocations, and even the overall genetic instability typical of cancer, may have their basis in changes in nuclear matrix protein composition that accompany the development of cancer. Given that the nuclear matrix plays critical roles in DNA organization and gene expression, changes in nuclear matrix structure would result in altered DNA topology and a change in interaction of various genes with the matrix, which could in turn set off a cascade of events leading to cancer. The proven association of genes such as the retinoblastoma susceptibility gene with p84, a nuclear matrix protein [Durfee et al., 1994] and the MAR binding activity of the protein p114, which can only be demonstrated in breast cancer cells [Yanagisawa et al., 1996] support such a possibility. Further study is required to better establish the precise chronology of the cancer initiation process to ascertain the role of the nuclear matrix in this series of events with accuracy.

THE NUCLEAR MATRIX IN NEOPLASTIC TRANSFORMATION

Most nuclear matrix proteins are found to be common to most cells, whereas some are both cell and tissue type specific [Fey and Penman, 1988]. Twenty-five of the most abundant NMPs are found to comprise 75% of all the nuclear NMPs. These proteins are common to all cells [Mattern et al., 1997]. We previously demonstrated that the protein composition of the nuclear matrix is tissue specific and can serve as a "fingerprint" of each cell and/or tissue type [Getzenberg, 1994]. Mitogenic stimulation and the induction of differentiation alter the composition of nuclear matrix proteins and structure [Dworetsky et al., 1990]. The composition of the nuclear matrix is also found to change concomitantly with the neoplastic transformation of the cell. This association is best understood when analyzed with respect to nuclear shape. It is evident that one of the most consistent pathologic features of a neoplastic cell is its altered nuclear shape with or without an accompanying increase in the nuclear-cytoplasmic ratio. Since the nuclear matrix is considered responsible for maintaining nuclear shape, an alteration in nuclear shape should involve a change in the nuclear matrix protein composition. The nuclear matrix proteins are also altered by other factors, including exogenous steroids such as estradiol, testosterone, and vitamin D [Konety et al., 1999] (B.R. Konety and R.H. Getzenberg, unpublished observations). Tissues such as the prostate contain specific binding sites for dihydrotestosterone on the nuclear matrix. These binding sites can be modulated in response to the hormonal status of the animal [Barrack, 1993].

Neoplastic transformation of a cell results in a comprehensive change in the morphology and nuclear architecture of the cell. These characteristic changes have been exploited in the diagnosis of many types of cancer. Features that have proved useful in identifying cancer cells are altered nuclear morphology, nuclear morphometry, variations in patterns of lamin expression, and the unique nuclear matrix protein composition of specific tumors. The cell type-specific alteration of nuclear matrix protein composition in the process of neoplastic transformation has led to the analysis of nuclear matrix protein composition of a variety of tumors in an effort to determine whether these proteins can be developed as diagnostic or prognostic markers for cancer.

LAMINS AND CANCER DIAGNOSIS

The network of filaments that make up the nuclear lamina are composed of two types of polypeptide filaments—the type A and type B lamins with type A lamin, including both types A and C, as they are related transcripts from a single gene [Fisher et al., 1986]. Type B lamins are composed of subtypes B1 and B2 [Hoger et al., 1990]. Lamin expression varies with stage of differentiation even in normal cells. The expression of lamins is found to be significantly altered in undifferentiated neoplastic cells. Neoplastic transformation with the attendant loss of differentiation leads to the decrease or loss of expression of specific types of lamin in the nuclei of cancer cells. Lamin B appears to be expressed predominantly in undifferentiated embryonal carcinoma cells and in lung cancers [Lanoix et al., 1992; Kaufman et al., 1991], whereas higher expression of lamin A is found in nonproliferative tissue [Coates et al., 1996]. This finding suggests that there is selective loss

of lamin A expression in tumor tissues. However, the fact that the lamins are present in all cell types with a lack of any cell or tissue specificity makes them poor candidates for diagnostic markers. Because of their lack of specificity, they can only be used to distinguish normal tissue from tumor at best. The many other cell surface and cytoskeletal markers available render the lamins redundant even for this purpose.

NUCLEAR MATRIX IN CANCER DIAGNOSIS

The initial indication that NMPs might be altered in cancer cells came from the studies of Berezney and colleagues [1979], who demonstrated characteristic alterations in hepatoma cells. Studies were also performed demonstrating that different cancer cell lines could be distinguished from each other based on their NMP patterns [Fey and Penman, 1988]. Using a unique approach, we examined the NMP composition of the normal and transformed prostate. Distinctive differences in NMP composition were found between normal rat prostate and cultured rat prostate tumor cells derived from the Dunning R3327 cell line [Getzenberg et al., 1991]. Using high-resolution two-dimensional gel electrophoresis, 10 proteins were identified in the normal rat dorsal prostate (site of origin of the Dunning tumor), which were absent in each of the three Dunning tumor cell lines tested (G, AT2, MLL). Three proteins were found in each of the three tumor cell lines, which were absent in all the normal rat prostate tissue. Differences in protein composition were also identified between the tumor cell lines, with two proteins present only in the less aggressive and nonmetastatic G cell line (G1, G2), and two proteins identified exclusively in the metastatic AT-2 and MLL cell lines (AM1, AM2). Similar analysis of human normal prostate, benign prostatic hyperplasia, and prostate cancer tissue demonstrated significant differences in NMP composition among them [Partin et al., 1993]. One NMP identified as PC-1 was found only in the prostate cancer tissue specimens and was absent in the other tissues. PC-1 has been detected using a monoclonal antibody (PRO:4-216) in frozen tissue sections from 85% of 22 prostate cancer patients, 5% of tissues from patients with benign prostatic hyperplasia and 9% of normal prostate tissue sections analyzed [Partin et al., 1997]. Sequencing of protein spots recognized by this antibody demonstrated that the protein was nucleophosmin, an RNA-associated nuclear phosphoprotein present more abundantly in actively proliferating cells [Subong et al., 1999]. Further analysis of nuclear matrix protein expression in tissue from patients with different stages of prostate cancers showed that an additional protein, YL-1, is present in all the tumors with poor prognosis and only in 3 of 10 cancers with a good prognosis [Lakshmanan et al., 1998]. This protein was absent in normal prostate tissue specimens.

Khanuja et al. [1993] identified four different nuclear matrix proteins that were present only in breast cancer tissue and absent in normal breast tissue. Yanagisawa et al. [1996] have identified a 114-kD protein that is present only in breast cancer tissue. This protein was present in 43 of 43 specimens of breast cancer tissue analyzed and was absent in adjacent normal breast tissue. The protein was also not identifiable in the other benign breast conditions such as fibroadenoma, fibrocystic disease, or atypical hyperplasia. This protein has also been shown to bind MAR, which lends it a potential functional significance.

Donat et al. [1996] found two proteins in laryngeal carcinoma tissue and in the Hep2 laryngeal carcinoma cell line that were absent in samples of normal laryngeal epithelium. In comparing normal tonsil tissue with primary tonsillar carcinoma and metastatic tonsillar carcinoma tissue, these investigators discovered four proteins that were present only in the cancers. A different set of four NMPs were also identified in oral cancer tissue, which were absent from normal oral tissue samples. Similar differences in NMP composition between benign and malignant tissue have been found when comparing squamous cell carcinoma of the head and neck [McCaffrey et al., 1997].

Other tumors for which similar differences in NMP composition have been identified include renal carcinoma [Konety et al., 1998], rat osteosarcoma [Bidwell et al., 1994], human colon carcinoma [Keesee et al., 1994], and human cervical carcinoma [Yang, et al; 1997; Miller et al., 1992]. A description of the tumors whose nuclear matrix protein composition has been analyzed and the unique proteins that have been discovered are shown in Table I.

BLADDER CANCER-SPECIFIC NMPS

The application of nuclear matrix proteins to clinical practice for the diagnosis/prognosis of tumors has been shown in our recent studies in

TABLE I. Nuclear Matrix Proteins Associated With Various Neoplasms

Investigators	No. of samples (n)	Tissue type	Name of protein(s)		Cell lines analyzed
			Normal specimens	Cancer specimens	
Getzenberg et al. [1991]	N/A	Dunning rat prostate cancer	ND1–ND10	D1, D2, D3	G, AT2, AT3, MLL
Getzenberg et al. [1991]	N/A	Dunning prostate tumor cell lines	—[a]	G cells—G1, G2 AT-2, MLL cells—AM1, AM2	—
Partin et al. [1997]	21	Human normal prostate, BPH, and cancer tissues	—	PC1	—
Laksmanan et al. [1998]	39	Human prostate cancer	—	Poor prognosis tumors—YL1	—
Keesee et al. [1994]	18	Human colon cancer	NC1–NC4	CC1–CC6	DLD, HT-29, LoVo, COLO, Caco, SW-1116
Khanuja et al. [1993]	10	Human breast cancer	NMNB-A, NMNB-B	NMBC-W, NMBC-X, NMBC-Y, NMBC-Z	MCF-10 (mortal), MCF-10A, H-RAS-MCF10A, C-NEU-MCF10A
Yanagisawa et al. [1996]	43	Human breast cancer	—	p114	MCF-10A, ZR-75-1, SK-BR-3, MDA-MB-231
Donat et al. [1996]	9	Human squamous cell carcinoma of the head and neck	—	A,B	Hep-2
McCaffery et al. [1997]	12	Human squamous cell cancer of head and neck	N12–N15	C1–C11	None
Getzenberg et al. [1996]	17	Human bladder transitional cell carcinoma	BLNL1–BLNL3	BLCA1–BLCA4	T-24, UMUC2, 253j
Konety et al. [1998]	17	Human renal cell carcinoma	RCNL1	RCCA1–RCCA5	A498, 769-P
Konety et al. [submitted]	24	Human bladder cancer	—	BLCA-4	—
Miller et al. [1992]	12	Colon, bladder, breast, lung, ovarian, endometrium, prostate, and rectal cancers	—	29-7NMP, 22-18 NMP	ME-180

NA, not applicable.
[a]None studied or none found.

bladder cancer. We have been successful in identifying six nuclear matrix proteins (BLCA-1 to 6) in samples from 24 patients with transitional cell carcinoma of the bladder that are absent in the adjacent normal bladder tissue obtained from the same patients, all of whom underwent total bladder excision [Getzenberg et al., 1996]. The patients were of varying ages, and the tumors were of different grades and stages. We also found three proteins that are present only

in the normal bladder tissue and not in the tumor tissue (BLNL1, 2, and 3). The cancer-specific NMPs were also not identifiable in normal bladder tissue obtained from organ donor bladders. Although we have now sequenced many of these proteins, we have focused much of our work on the bladder cancer-specific NMP BLCA-4. Protein sequence analysis of BLCA-4 indicates that it has some homology with two nonvertebrate proteins giving us no information about its functional role. We have been able to generate antibodies against BLCA-4 by immunizing rabbits with peptides encoding it. These antibodies have been able to detect BLCA-4 by immunoblot in nuclear matrix extracts of human bladder tumor tissue and in adjacent "normal" tissue from individuals with bladder cancer, but not in normal organ donor bladder tissue [Konety et al., submitted]. The BLCA-4 protein is also identifiable by immunohistochemistry demonstrating punctate nuclear staining of the tumor cells.

Recently, using an immunoassay, we have been able to detect BLCA-4 in the urine of patients with bladder cancer. This urine-based immunoassay is able to differentiate individuals with bladder cancer from those without the disease. Although the assay has a low background level, the resulting specificity of this assay is 100%, whereas the sensitivity is 96.4% [Konety et al., submitted]. If this level of sensitivity and specificity continues to be supported by larger clinical trials, this will become the most specific tumor marker yet developed.

Another important understanding emerging from these studies is that they indicate that BLCA-4 is expressed throughout the bladder in patients with bladder cancer. The fact that this protein is expressed in morphologically normal areas of the bladder supports the existence of a field effect in bladder cancer. Furthermore, BLCA-4 could be altered early during the course of neoplastic transformation. In order to test whether BLCA-4 is indeed an early marker of the disease, we investigated expression in an animal model of bladder cancer [Steinberg et al., 1990]. These data indicate that BLCA-4 is indeed expressed before the observation of grossly visible tumors. All these data suggest that the bladder cancer-specific NMP, BLCA-4, is expressed in bladder cancer tissue from various sources and of various grades and stages. It is also released into the urine, hence its presence can be detected by urine-based assays. This presents a unique opportunity for the use of this NMP as a marker for diagnosing and monitoring patients with bladder cancer and clearly shows that NMPs can serve as exciting new tumor markers with unique properties.

A Food and Drug Administration (FDA)-approved, NMP-based test is commercially available that can be used in monitoring for bladder cancer. This assay, called NMP22, is based on a different NMP than BLCA-4. The assay detects the nuclear mitotic apparatus (NuMA) protein in the urine of patients with bladder cancer [Keesee et al., 1996]. Using the NMP22 assay, a greater number of patients who are at risk for imminent recurrence of cancer can be identified accurately compared with other standard diagnostic methods currently in use [reviewed in Pirtskhalaishvili et al., 1999]. However, an elevated urinary NMP22 value may not signify bladder cancer recurrence in all cases, especially in patients who have concomitant bladder inflammation. The NuMA protein is present in all cells, including noncancerous cells, which implies that its presence in the urine, per se, is not necessarily indicative of cancer, but that it would be present in higher levels in patients with bladder or other tumors, as well as other confounding or coexisting conditions. The use of cancer-specific NMPs such as BLCA-4 will present an advantage in this situation wherein the diagnostic accuracy can be significantly enhanced.

The role of nuclear matrix proteins in the diagnosis of cancer is in its evolution. The excitement related to their initial discovery in the various cancers is justified, given their apparent high specificity. Subsequent development of antibody-based tests to detect these proteins in tissue and body fluids demonstrates the fact that they are released from tumor cells, making them amenable to detection in vivo. Studies indicate that these proteins are recoverable and detectable in the serum and urine of cancer patients [Miller et al., 1992; Getzenberg et al., 1996]. We have found that our initial studies using the urine of bladder cancer patients has borne out the high level of specificity that was evident in the initial examination of tissue specimens. However, the presence of other NMPs, such as NuMA, which are detectable by the NMP22 test in the urine of bladder cancer patients and in the blood of patients with other types of cancers [Miller et al., 1992], indicates that NMPs of various types are released from

tumors and can be detected. Some of these NMPs are present in most cell types and, although their presence in excessive amounts in body fluids may indicate the presence of malignancy, it may not signify the specific location or histologic type of tumor. In essence, the NMP22 and other such tests provide us with the "proof of principle" that detection of NMPs can be used as a basis to diagnose cancer, and their detection is possible by means of easily performed tests. It is important to develop tests using NMP targets specific to each tumor type to enhance the accuracy of these tests. NMP-based diagnostic tests can also be used to distinguish between different types of tumors when the distinction cannot be made accurately on histologic appearance alone [Hughes and Cohen, 1999]. Tests that permit the detection of cancer-specific nuclear matrix proteins in body fluids such as effusions, cerebrospinal fluid (CSF), and inflammatory exudates, can significantly facilitate establishment of a diagnosis in situations in which standard cytologic testing is inadequate. The fact that nuclear matrix proteins can be detected using antibodies in serum, urine, and even by flow cytometry further increases the applicability of the such tests to various situations.

Further understanding of the precise functional role of these cancer-specific NMPs will better elucidate the mechanisms of carcinogenesis in these tumors and perhaps provide us with additional targets for therapy. Future research will be directed toward exploiting the full potential of the alterations in NMP composition for cancer diagnosis and the identity and function of these molecules. We are approaching the final stages of development of a test for the diagnosis of bladder cancer, which will be based on detecting the BLCA-4 protein in the urine of patients suspected to have bladder cancer. We expect this test to be more accurate than the NMP22 test, even in patients with coexisting bladder conditions such as inflammation, which tend to render the NMP22 test inaccurate. Similar efforts are under way to develop a diagnostic test for prostate cancer, using antibodies to detect the presence of the prostate cancer specific NMPs in the serum of prostate cancer patients. The obvious superiority of urine-based tests for NMP detection over currently existing diagnostic modalities ensures that we will witness a rapid proliferation of these tests for various tumors. Characterization of some of these cancer-specific proteins will allow us to better define their role in the neoplastic process and may also yield dividends in terms of more accurate tests or an improved delineation of the precise context in which these tests could and should be used. A central deficiency in tumor markers is their historically low specificity for the disease in question. Using NMPs as a marker provides an opportunity to increase the specificity of the marker for the specific cancer type. Without a more complete understanding of the role of the cancer-specific proteins in the cancer cell, it would also be difficult to develop therapies employing these proteins as targets. The identification of the NMPs unique to various cancers has provided us with many new avenues for exploration that we anticipate and eventually provide us with many new tools in the battle against cancer.

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