ARTICLE
Journal of Cellular Biochemistry 108:1274–1279 (2009)

Journal of Cellular Biochemistry

Identification of Nuclear Structural Protein Alterations Associated With Seminomas

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ABSTRACT

Currently, there are no specific markers available for the early detection and for monitoring testicular cancer. Based upon an approach that targets nuclear structure, we have identified a set of proteins that are specific for seminomas, which may then have clinical utility for the disease. Utilizing samples obtained from men with no evidence of testicular cancer (n = 5) as well as those with seminomas (n = 6), nuclear matrix proteins were extracted and separated using a high-resolution two-dimensional electrophoresis gel system. The proteins were identified by mass spectrometry analysis. These analyses revealed seven nuclear matrix proteins associated with the normal testes, which did not appear in the seminomas. In the seminomas, four nuclear matrix proteins were identified to be associated with the disease that were absent in the normal testes. Mass spectrometric and immunoblot analyses of these proteins revealed that one of the proteins identified in the normal testes appears to be StAR-related lipid transfer protein 7 (StARD7). In the non-seminoma tissues, one of the identified proteins appears to be cell division protein kinase 10 (CDK10). Both StarD7 and CDK10 could potentially be involved in cell differentiation and growth, and thus may serve as potential targets for therapy of prognostication of seminomas. This is the first study to examine the role of nuclear structural proteins as potential biomarkers in testicular cancer. We are currently examining the roles of some of the identified proteins as potential biomarkers for the disease. J. Cell. Biochem. 108: 1274–1279, 2009.

KEY WORDS: TESTICULAR CANCER; SEMINOMAS; PROTEOMICS; BIOMARKERS; NUCLEAR MATRIX

Testicular germ cell cancer represents about 1% of male cancers and is accountable for approximately 0.1% of cancer-related mortality in men [Jemal et al., 2009]. In 2009, the American Cancer Society estimates that there will be 8,400 new cases and 380 related death for testicular cancer [Jemal et al., 2009]. Histologically, testicular germ cell cancer is divided into two major subgroups: seminomas and non-seminoma germ cell tumors. Seminomas account for 50% of testicular cancer, and they arise most frequently in the fourth decade of life, whereas non-seminoma germ cell tumors comprise 40% of testicular cancer, and occur most frequently in the third decade of life. The remaining 10% of testicular cancer are combined tumors and they typically contain both seminoma and non-seminoma elements. The disparity between seminoma and non-seminoma germ cell tumors is essential for the purpose of treatment and prognostication.

Over the last 40 years, the incidence of testicular cancer has doubled in Europe for unknown reasons [Huyghe et al., 2007]. When diagnosed at early stage, testicular cancer is typically curable with a high survival rate. Nevertheless, there are no specific markers that can be used for early detection and to monitor the disease. Currently available markers for this disease include α-fetoprotein (AFP), hCG, and lactase dehydrogenase (LDH). However, these markers are not very specific and they are only detected in approximately 60% of men with testicular cancer [Trigo et al., 2000]. In addition, the sensitivity of these markers is limited, and the levels of these markers are usually "normal" in about 40% of men with disease recurrence [Trigo et al., 2000]. Therefore, the discovery of additional markers would clearly facilitate the management of men with testicular cancer.

One of the hallmarks of the cancer cell is alterations in the shape, size, and morphometry of the nucleus. In concordance with these changes are alterations in the composition of nuclear structural/matrix proteins. Alterations in nuclear matrix proteins have been identified in various cancers including breast, prostate, bladder, lung, ovarian, and squamous carcinoma of the head and neck [Konety and Getzenberg, 1999; Leman and Getzenberg, 2008]. Our

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Received 21 August 2009; Accepted 25 August 2009 • DOI 10.1002/jcb.22357 • © 2009 Wiley-Liss, Inc.
Published online 30 September 2009 in Wiley InterScience (www.interscience.wiley.com).

laboratory has utilized a focused proteomics approach to identify a set of proteins associated with changes in the nuclear structure/architecture. Assessment of these nuclear proteins resulted in identification of biomarkers that are specific for prostate, colorectal, and bladder cancers [Leman and Getzenberg, 2008]. These proteins have been successfully developed into both blood- and urine-based markers with high sensitivity and specificity [Konety et al., 2000; Myers-Irvin et al., 2005; Leman et al., 2007a,b, 2009].

In this study, we utilized a similar proteomics approach to identify a set of nuclear structural proteins that are specific for seminomas. We identified four nuclear matrix proteins that are specific for seminomas and seven nuclear matrix proteins that are specific for the normal testicular tissues. Identification of these proteins will potentially allow us to develop additional markers for the detection of testicular germ cell tumor (in particular seminomas), as well as determine ways in which changes in the nuclear structural composition can serve as potential prognostic targets for monitoring the disease.

MATERIALS AND METHODS

TISSUE SAMPLES

Testicular tissues were obtained from the Department of Pathology of the Johns Hopkins Hospital, Baltimore, Maryland, USA and from the Department of Urology of Charité Campus Benjamin Franklin, Berlin, Germany under institutional IRB approved protocol. The normal testes (n = 5) are obtained from men with no evidence of testicular cancer. Four of the normal testicular tissues were obtained from men with prostate cancer, and one tissue was obtained from a man diagnosed with Fournier's gangrene. The testicular cancer tissues are from men diagnosed with seminomas (n = 6). All tissue samples were from Caucasian men. Profiles of these samples are shown in Table I. Prior to nuclear matrix protein isolation, all tissues were stored at −80°C.

ISOLATION AND ANALYSIS OF NUCLEAR MATRIX PROTEIN COMPOSITION

The nuclear matrix proteins were isolated using the previously described methods [Getzenberg et al., 1991; Leman et al., 2002]. Protein concentration was determined by resuspending the proteins in PBS and using the Coomassie Plus protein assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin as standard. For gel electrophoresis, the ethanol precipitated NMPs were dissolved in sample buffer consisting of 9 M urea, 65 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 2.2% ampholytes, and 140 mM dithiorthreitol. The final pellet containing nuclear matrix proteins represented less than 1% of the total cellular proteins.

High-resolution two-dimensional electrophoresis was performed to separate the extracted proteins. Fifty micrograms of proteins were loaded onto each tube gel (1 mm × 18 inch). One-dimensional isoelectric focusing was carried out for 18,000 V-h after 1.5 h of prefocusing. The tube gels were then extruded and placed on top of 1 mm 10% SDS–Duracyl (Genomic Solution, Ann Arbor, MI) high-tensile strength PAGE slab gels. The slab gels were electrophoresed at 12°C constantly for 5–5.5 h. The gels were then fixed with 50% methanol and 10% acetic acid. After thorough rinsing and rehydration, the gels were treated with 5% glutaraldehyde and 5 mM dithiorthreitol after buffering with 50 mM phosphate (pH 7.2). The gels were stained with a silver stain kit (Protea Biosciences, Inc., Morgantown, WV) according to the manufacturer's protocol. Only spots clearly reproducibly observed in the gels were counted as representing nuclear matrix components. The identified protein spots were cut out, de-stained, and sent out for mass spectrometry analysis (Protea Biosciences, Inc.) for protein identification.

ANTIBODIES

Purified rabbit polyclonal antibody against cell division protein kinase 10 (CDK10; cat #AP7516, Abgent, San Diego, CA), chicken polyclonal antibody against StARD7 (cat #XW 8003, ProSci, Inc., Poway, CA), secondary anti rabbit antibody conjugated with horseradish peroxidase (Amersham Life Sciences, Arlington Heights, IL) and secondary rabbit anti chicken antibody (AbCam, Cambridge, MA) were used to validate some of the protein spots identified by mass spectrometry analysis.

ONE-DIMENSIONAL IMMUNOBLOT

Fifty micrograms of protein was loaded and separated by 4–20% SDS–PAGE along with a distinct lane containing 10 μl of Kaleidoscope Prestained Markers (Pierce). The proteins were then transferred to 0.22 μM nitrocellulose membranes (Bio-Rad, Richmond, CA) utilizing a semi-dry transfer apparatus (Bio-Rad). The membranes were incubated overnight in 5% non-fat dry milk in phosphate buffered saline (PBS) with 0.1% Tween-20. The membranes were then incubated with 1:100 dilution of primary

TABLE I. Patient Characteristics

Institution	Case	Age	Diagnosis	Size/stage	Metastases
CCBF	Normal testes	76	Fournier's gangrene, acute inflammation, reduced spermatogenesis	N/A	N/A
CCBF	Normal testes	68	Prostate cancer, benign testicular tissue, reduced spermatogenesis	N/A	N/A
CCBF	Normal testes	72	Prostate cancer, benign testicular tissue, reduced spermatogenesis	N/A	N/A
CCBF	Normal testes	64	Prostate cancer, benign testicular tissue, reduced spermatogenesis	N/A	N/A
CCBF	Normal testes	82	Prostate cancer, benign testicular tissue, reduced spermatogenesis	N/A	N/A
CCBF	Testicular cancer	57	Seminoma	33 mm × 27 mm × 22 mm, pT1, Stage I	None
CCBF	Testicular cancer	33	Seminoma	57 mm × 42 mm × 33 mm, pT1, Stage I	None
CCBF	Testicular cancer	47	Seminoma	80 mm × 55 mm × 48 mm, pT1, Stage I	None
JHH	Testicular cancer	25	Seminoma	45 mm diameter, pT1, Stage I	None
JHH	Testicular cancer	47	Seminoma	40 mm diameter, pT1, Stage I	None
JHH	Testicular cancer	49	Seminoma	15 mm diameter, pT1, Stage I	None

JHH, Johns Hopkins Hospital, USA; CCBF, Charité Campus Benjamin Franklin, Berlin, Germany.

antibody in PBS with 5% non-fat dry milk and 0.2% Tween-20 at room temperature for 1 h. The membranes were then washed for three times (10 min for each wash) with PBS and 0.2% Tween-20 and incubated for 1 h with horseradish peroxide-conjugated secondary antibody at a dilution of 1:5,000 at room temperature. The membranes were then washed again with PBS and 0.2% Tween-20 (three 10-min washes, followed by three quick washed, and two 10-min washes). The proteins were detected by a chemiluminescence reaction using the Super Signal West Dura chemiluminescence reaction kit (Pierce).

TWO-DIMENSIONAL IMMUNOBLOT

High-resolution two-dimensional electrophoresis was performed to separate the extracted proteins as described above. Briefly, 100 μg of NMPs were loaded onto each tube gel (1 mm × 18 inch). One-dimensional isoelectric focusing was performed for 18,000 V-h after 1.5 h of pre-focusing. The tube gels were then extruded and placed on top of 1 mm 10% SDS–Duracyl (Genomic Solution) high-tensile strength PAGE slab gels. The slab gels were electrophoresed at 12°C constantly for 5–5.5 h and then transferred to 0.22 μM nitrocellulose membranes (Bio-Rad). Thereafter, the same procedure described above for the one-dimensional immunoblot was performed for the two-dimensional immunoblot analysis.

RESULTS

Using high-resolution two-dimensional analyses, we initially compared the two-dimensional gel analysis patterns of the seminomas, since these tissues were obtained from two institutions (JHH, USA and CCBF, Germany). As shown by the representative two-dimensional gels of the nuclear structural protein from the seminomas obtained from the two different institutions (Fig. 1), the patterns were almost identical, and this was consistent in all the seminoma tissues. We next compared the nuclear structural protein composition between the normal testes and seminomas. As shown in Figure 2, analyses of the high-resolution two-dimensional gels showed that there were seven nuclear structural proteins (A, B, C, D, E, F, G, and H) that were present in the normal testes, but not in the seminomas. The analyses also showed that there were four protein spots (I, J, K, and L) that were present in the seminomas, but absent in the normal testes (Fig. 2).

To begin to elucidate the identities of these proteins, we collected spots from the two-dimensional gels for mass spectrometry analyses. As shown in Table II, mass spectrometry analyses of these proteins revealed that some of the proteins identified in the normal testes appear to be Testis specific Y-encoded-like protein 4 (spot D), cytokeratins (spots A, C, and G), glutamine synthetase (spot F), and StAR-related lipid transfer protein 7 (spots B and E). In the seminoma tissues (Table III), the majority of the identified protein spots (spots I, J, and K) appeared to be gamma-tubulin complex component 6 (GCP6), whereas one protein spot (spot L) appeared to be CDK10.

We next obtained commercially available antibodies to validate the mass spectrometry analyses results. At the time when these studies were performed, our laboratory was able to find two commercially available antibodies against three of the protein spots identified in both normal testes (spots B (MW 39 kDa, pI 5.8) and E (MW 42 kDa, pI 6.6), which correspond to StarD7 (StAR-related lipid transfer protein 7)) and seminoma tissues (spot L (MW 45 kDa, pI 5.7), which corresponds to CDK10). We performed both one-and two-dimensional immunoblots on the nuclear matrix protein extracts using commercially available antibodies against StarD7 and CDK10. As shown in Figure 3A, antibody against StARD7 detected a protein band at a MW of 35 kDa in the nuclear matrix protein extracts from the normal testes, but not in the seminoma tissues. Antibody against CDK10 picked up protein bands in the MW range of 38–42 kDa in the nuclear matrix protein extracts from the seminomas, but not in the normal testes (Fig. 3A). Two-dimensional immunoblot analyses on the seminoma tissues further confirmed that antibody against CDK10 was able to pick up the proteins spot L in the two-dimensional gels at MW of 45 kDa and pI of 5.7 (Fig. 3B).

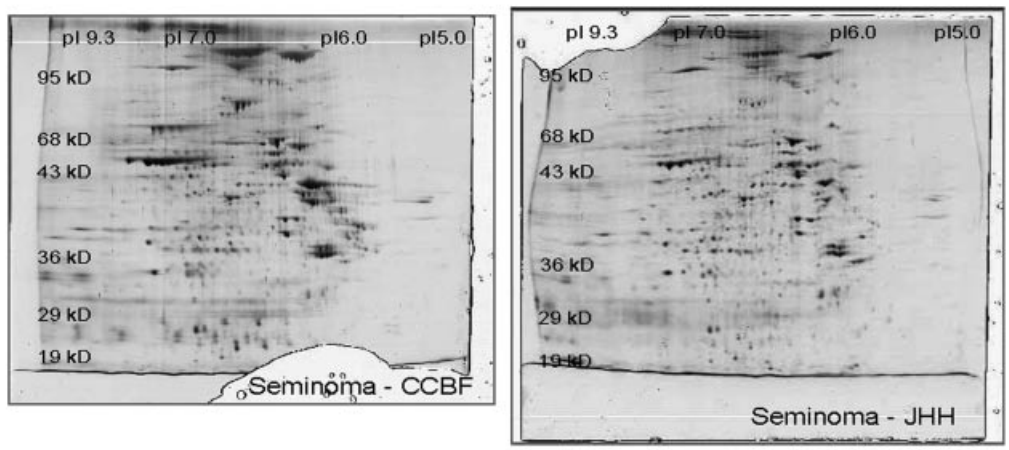

Fig. 1. Representative two-dimensional gels of nuclear structural protein composition of the seminoma tissues from two different institutions: JHH (Johns Hopkins Hospital, Baltimore, MD, USA) and CCBF (Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany).

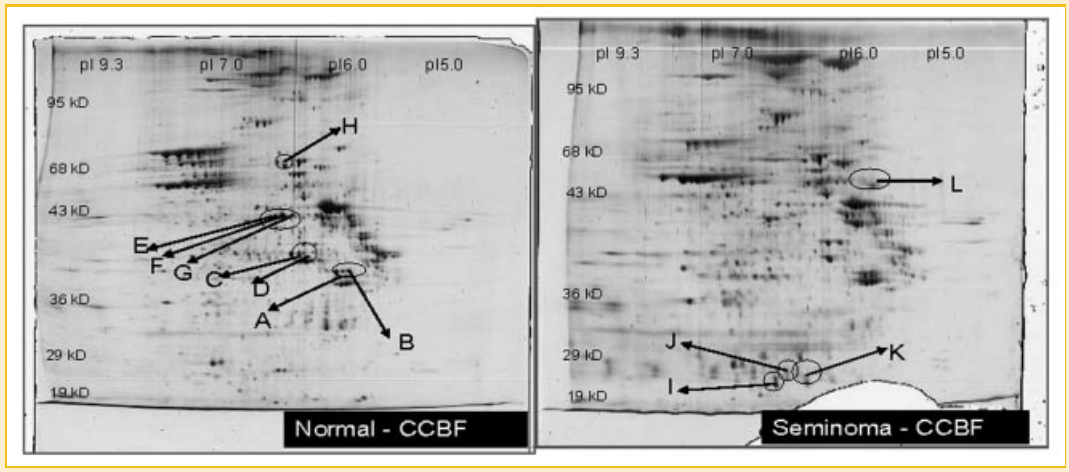

Fig. 2. Representative two-dimensional gels of nuclear structural protein composition of the normal testes and seminoma tissues. Seven nuclear structural proteins (A, B, C, D, E, F, G and H) are present in the normal testes, but not in the seminomas. Four protein spots (I, J, K and L) are present in the seminomas, but absent in the normal testes.

DISCUSSION

Although testicular cancer is typically curable with a high-survival rate, there are no specific markers that can be used for either early detection or to monitoring the disease. Currently available markers such as AFP, hCG, and LDH are not very specific and they are only detected in approximately 60% of men with testicular cancer [Trigo et al., 2000]. In addition, the sensitivity of these markers is limited, and the levels of these markers are usually "normal" in about 40% of men with disease recurrence [Trigo et al., 2000]. Therefore, the discovery of additional markers would clearly facilitate the management of men with testicular cancer. Recently, cell-free circulating mitochondrial DNA in the sera of patients with testicular cancer has been shown to be a novel non-invasive diagnostic biomarker for monitoring the disease [Ellinger et al., 2009]. Although the mitochondrial DNA levels were higher in men with testicular cancer, they did not correlate with any of the clinicopathological variables such as stage, lymph node invasion, and clinical stage [Ellinger et al., 2009]. Therefore, ongoing discovery of testicular cancer biomarker(s) is crucial for management of the disease.

Our laboratory has previously identified nuclear structural proteins that are specific for prostate, colorectal, and bladder cancers. Using a similar approach, we have identified a set of nuclear structural proteins that are specific for seminomas, which may then be utilized for the detection and monitoring of the disease. In this study we identified seven proteins that were specific for the normal testes and four proteins that were specific for seminomas. Identification of these protein spots by mass spectrometry analyses and validation studies by immunoblot analyses revealed that one of the protein spots that was present in the normal testes but absent in the seminomas was to StarD7 (StAR-related lipid transfer protein 7). StarD7 is a protein that belongs to the family of StAR1-related lipid transfer (START) proteins [Ponting and Aravind, 1999; Tsujishita and Hurley, 2000]. These START proteins have been shown to be involved in lipid transport, metabolism and signaling [Ponting and Aravind, 1999; Tsujishita and Hurley, 2000]. Expression of StarD7 has been reported in human choriocarcinoma cell lines JEG-3 and JAR, as well as in colorectal carcinoma HT29 and hepatocellular carcinoma HepG2 cells [Rena et al., 2009]. In addition, increased StARD7 protein expression and subcellular relocalization have also been reported in differentiating cytotrophoblast, suggesting that it may play a functional role in the process of throphoblast differentiation through phospholipids uptake and transport [Angeletti et al., 2008].

Mass spectrometry analyses on the protein spots identified in the nuclear matrix of the normal testes revealed that two proteins (spots B (MW 39 kDa, pI 5.8) and E (MW 42 kDa, pI 6.6)) could be StarD7 protein. Furthermore, our immunoblot analyses showed that antibody against StARD7 picked up a protein band at a MW of 35 kDa in the nuclear matrix protein extracts from the normal testes. Two putative human StarD7 isoforms of 34.4 and 43.9 kDa (Acession

TABLE II. Nuclear Matrix Proteins From Normal Testicular Tissues

Protein spots	MW (kDa)	pI	Mascot PMF results	Score	Peptide sequence coverage (%)	Predicted MW (kDa)	Predicted pI
A	39	6.0	Cytokeratin 19 (CK 19)	28	50	44	5.04
B	39	5.8	StAR-related lipid transfer protein 7 (StARD7)	29	20	35	5.79
C	40	6.3	Cytokeratin 18 (CK 18)	43	56	48	5.34
D	40	6.2	Testis specific Y-encoded-like protein (TSYL4)	25	50	45	8.79
E	42	6.6	StAR-related lipid transfer protein 7 (StARD7)	34	25	35	5.79
F	42	6.5	Glutamine synthetase (GLNA)	38	46	42	6.43
G	42	6.5	Keratin, type II cytoskeletal 7 (K2C7)	74	68	51	5.50

TABLE III. Nuclear Matrix Proteins From Seminoma Tissues

Protein spots	MW (kDa)	pI	Mascot PMF results	Score	Peptide sequence coverage (%)	Predicted MW (kDa)	Predicted pI
I	20–21	6.6	Gamma-tubulin complex component 6 (GCP6)	55	4	200	5.89
J	21–22	6.5	Gamma-tubulin complex component 6 (GCP6)	46	4	200	5.89
K	21	6.3	Gamma-tubulin complex component 6 (GCP6)	50	3	200	5.89
L	45	5.7	Cell division protein kinase 10 (CDK10)	52	18	41	9.06

number Q9NQZ5 and NP_064536, respectively) have been documented in the NCBI database [Angeletti et al., 2008]. Our findings suggest that the spots that were found in the normal testes could indeed be the isoforms of StarD7, and to our knowledge this is the first report that StarD7 protein may be associated with the nuclear matrix. However, it is unclear why StarD7 is absent in the seminoma tissues, but present in the nuclear matrix of the normal testes. We speculate that the presence of StarD7 in the normal testes

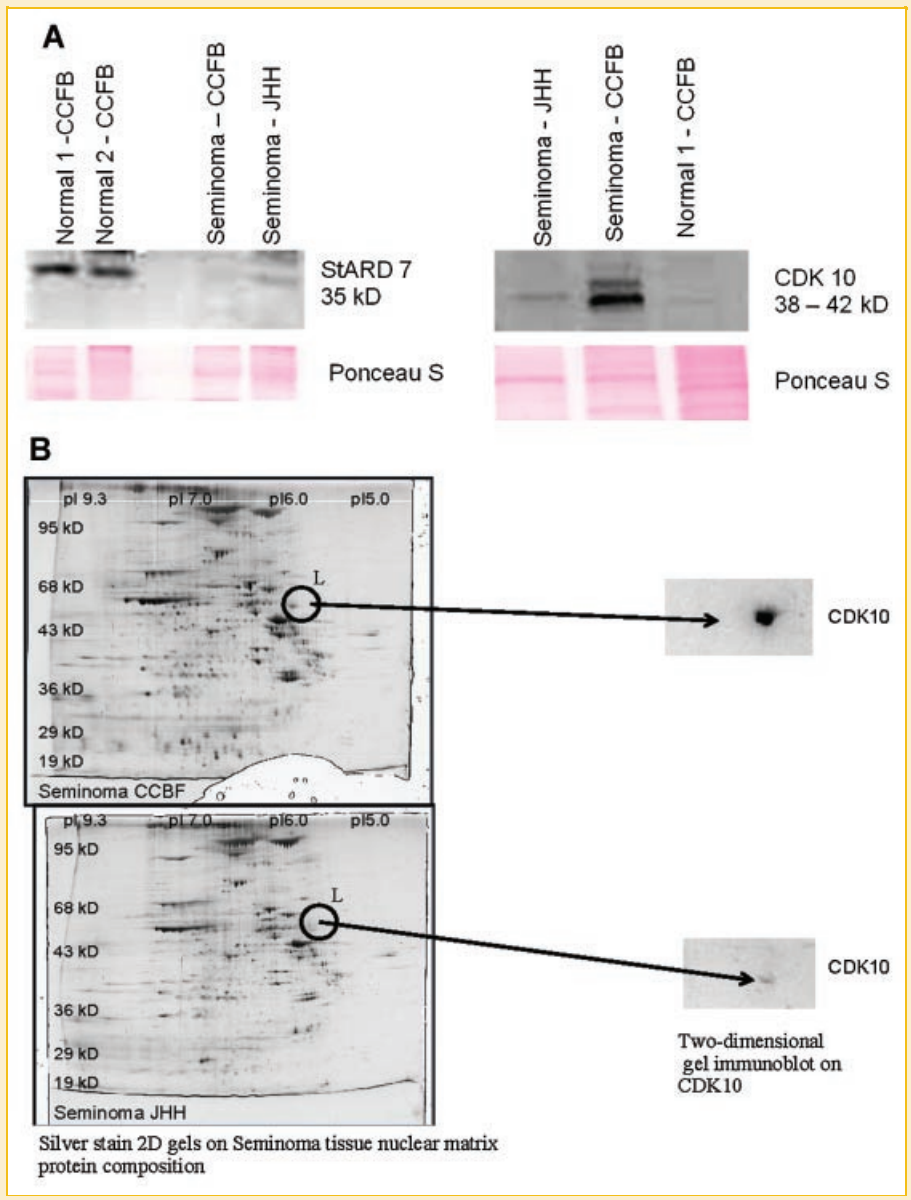

Fig. 3. Immunoblot analyses for StarD7 and CDK10 from the nuclear matrix protein extracts of the normal testes and seminomas. A: One-dimensional immunoblot analyses on StarD7 and CDK-10. B: Two-dimensional immunoblot analyses on CDK-10 on the seminoma tissues. JHH: Johns Hopkins Hospital, Baltimore, MD, USA; CCBF: Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany.

could play a role in maintaining the differentiated form of the normal cells and the loss of this protein could lead into malignancy.

Another protein that was found to be specific for seminomas but not found in the normal testes appeared to be CDK10. In our study, mass spectrometry and immunoblot analyses of protein spot L found in the nuclear matrix of seminomas showed that this spot could indeed be CDK 10. CDK 10 is a cdc2-related kinase that contains the Tyr and Thr residues present in all protein kinases [Bagella et al., 2006]. Two isoforms of human CDK10 have been reported: hcdk10-1 and hcdk10-2 [Crawford et al., 1999]. Although little is known about the role of this protein, hcdk10-1 has been reported to bind to Ets2 transcription factor, thus modulating its transactivation activity [Kasten and Giordano, 2001]. On the other hand, hcdk10-2 has been proposed to be involved in the G2/M transition of the cell cycle [Li et al., 1995]. Our immunoblot analyses showed that antibody against CDK10 picked up protein bands in the MW range of 38–42 kDa in the nuclear matrix protein extracts from the seminomas, but not in the normal testes. It is possible that both of the CDK10 isoforms are present in the nuclear matrix of the seminoma tissues. Although the exact role of this protein is not clear in testicular cancer, we speculate that CDK10 may play a role in the cell cycle regulation that may lead to malignancy. We are currently investigating the potential role of CDK10 in testicular cancer.

Taken together, we have shown that there are potentially two proteins that may play a role in the development of testicular cancer, in particular the seminomas. Identification of these nuclear structural proteins may enhance our understanding of the molecular changes associated with testicular cancer, in particular the seminomas. Both StarD7 (present only in the normal testes) and CDK10 (present only in the seminomas) could potentially be involved in cell differentiation and growth, and thus may serve as potential targets for therapy of prognostication of seminomas. One major limitation in this study is that we did not examine the nuclear structural components of the non-seminoma tissues. It is possible that the proteins identified in the seminoma tissues may also present in the non-seminoma tissues. Future studies including identification of nuclear structural proteins in the non-seminoma tissues as well as in testicular cancer cell lines are clearly warranted. This information can then be utilized in combination with other available markers for testicular cancer in order to facilitate the management of the disease.

ACKNOWLEDGMENTS

This study was supported through the Patana Fund of the James Buchanan Brady Urology Institute of The Johns Hopkins University School of Medicine.

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