CHAPTER V:

Characterization of Sperm Protein 17 in Human Somatic and Neoplastic Tissue

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1. Abstract

The Sp17 gene has been detected in multiple non-testes and several highly proliferating tissues, including rhuematoid arthritis and malignant neoplasias. Because the role of Sp17 in these tissues is unknown, the characterization of the Sp17 gene in diseased tissues may provide further insight into the regulation and function of Sp17. The Sp17-1 transcript was examined in multiple normal human tissues and cancer cell lines by RT-PCR. Moreover, both alternative transcriptional start sites, Sp17-1a and Sp17-1b, were detected in cancer cell lines. Similarly, a Sp17-2 transcript was detected by RT-PCR. Furthermore, northern blot analysis revealed Sp17 mRNA expression in all cancer cell lines examined. However, western blot analysis did not detect the Sp17 protein in these cells.

2. Introduction

Gene expression is frequently different in neoplastic cells, as compared to normal tissues. In particular, normal testicular proteins have been identified in a variety of malignant neoplasias. Recently, these proteins have been recognized as a group of tumor-specific antigens called cancer-testis (CT) antigens (1). CTs are characterized by 1.) mRNA expression predominantly in testis, 2.) gene activation and mRNA expression in multiple human tumors, 3.) existence of multiple gene families, and 4.) localization of coding genes to chromosome X (2). Examples of CT antigen families include MAGE, BAGE and GAGE, which were discovered by cloning cytotoxic T lyphocyte-recognized antigens expressed in melanoma cells. In addition, the CTs from melanoma (SSX2), esophageal cancer (NY-ESO-1) and renal cancer (SCP-1) were identified by the serological analysis of recombinant cDNA expression libraries (SEREX) (2).

CTs have been studied and detected in cancers including those of the brain, liver and lung (3, 4, 5). However, CTs expression was not detected in normal non-testis tissues (6). Thus, the selectivity of CT expression implicates CTs as a potential immunotherapeutic target in malignant neoplasias (1).

Sp17 exhibits several of the hallmarks of a CT antigen. Sp17 is a highly antigenic, testes specific protein whose known function is to bind sperm to the zona pellucida (7). In particular, a study of Sp17 in normal human tissues revealed that Sp17 mRNA is abundantly expressed in normal testis tissue (8, 9). In addition, Sp17 gene transcription, although in low abundance, has been described in normal tissues including human testes, sheep mucosa-associated lymphoid tissues and in cynomologous macaque esophagus, kidney, pancreas, small intestine, spinal column, spleen and thyroid (10, 11, Chapter 4). However, Sp17 mRNA expression and translation was not detected in these tissues. Hence the role of Sp17 gene transcription in these tissues is not known.

In addition to normal tissues, the Sp17 gene was previously described in human rheumatoid arthritis (RA) synovium as opposed to osteoarthritis synovium (12). Similarly, Sp17 mRNA expression was also detected in the metastatic stage, but not the transitional

phases, of a murine model of squamous cell carcinoma (13). Moreover, Sp17 was detected in multiple myeloma and implicated as a potential CT antigen (14). However, Sp17 has yet to be confirmed as a CT antigen. Thus, because RA and cancer cells are characterized, in part, by over proliferating cells, Sp17 may have a pathogenic role in cell migration, cell signaling and/or cell proliferation. Therefore, further investigation of Sp17 transcription and translation in diseased and highly proliferating tissues merits attention.

Furthermore, several models of cancer-associated genes have described the use of alternative transcriptional start sites, one benign and one pathogenic (15). In these models, in response to stimuli, one gene is typically upregulated while the other is downregulated (15, 16). For example, epithelial protein lost in neoplasm (EPLIN) is a protein that co-localizes to the actin stress fibers and focal adhesion plaques (16). The EPLIN gene has two isoforms; EPLIN-alpha and the EPLIN-beta. Recently, it was discovered that in human breast and prostate cancer cell lines, the expression of EPLIN-alpha is reduced, while the expression of EPLIN-beta is either unchanged or up-regulated. However, whether this mechanism occurs in Sp17, with alternative transcriptional start sites or with the Sp17-2 variant, has yet to be elucidated.

Cancer cell lines were used as a model to characterize the Sp17 gene in neoplastic tissues as compared to normal human somatic tissues. RT-PCR analysis was used to detect the Sp17-1 transcript and differentiate between alternative transcriptional start sites, Sp17-1a and Sp17-1b. Similarly, PCR was used to elucidate the Sp17-2 gene variant and RT-PCR was employed to detect potential Sp17-2 transcripts. Moreover, northern blot and western blot analyses was used to detect Sp17 mRNA expression and Sp17 translation in these cell lines.

3. Materials and Methods

3.1 Cell culture

Cancer cell lines (American Type Culture Collection, Manassas, VA) were donated as a gift to our laboratory. The cancer cell lines included HaCat (basal cell carcinoma); A459, Calu 1 (lung cancer); LuCaP, Du 145, PC3 (prostate cancer); 4C8 Glioma (glioma); Hela S3 (adenocarcinoma, cervix); 143B thymidine kinase (TK)-deficient (osteosarcoma).

The human HeLa S3, A549, and 143B TK-deficient (TK^-) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), at 37°C in 5% CO_2. In addition, 143B TK^- cells were grown in the presence of 25ug/ml of 5-bromo-2’-deoxyuridine (BrdU). The HaCat, Calu 1, LuCaP, Du 145, PC3 and 4C8 Glioma cells were grown under similar conditions until confluent.

3.2 RNA isolation, DNase treatment and Reverse transcription (RT)

Normal human somatic tissue samples were obtained from the University of California, Davis (UCD) Donated Body Program (UCD biological use authorization #0657). The human tissues examined included the adrenal, bladder, esophagus, colon,

kidney, liver, lung, lymph node, muscle, nerve, ovary, polymorphonuclear blood cells (pmbcs), parathyroid, spleen, spinal column, RA synovium and adult testes.

The human tissues and cultured cells were homogenized (50-100 mg or $5x10^6$ cells) in TRIzol reagent and the total RNA was extracted, analyzed and stored as described in Chapter 1, section 3.1. The total RNA extracts (3ug) were treated with DNase I and converted into cDNA by reverse transcription (RT) as described in Chapter 1, section 3.2 (Chapter 1, Table 1.1).

3.3 Polymerase chain reaction (PCR)

The Sp17 nucleotide sequence was amplified from various cancer cell line cDNAs by RT-PCR. Sets of Sp17 specific primers were created to detect the Sp17 transcript and distinguish between the Sp17 transcript variants; Sp17-1a and Sp17-1b (Table 4.1). In addition, Sp17 specific primers were used to amplify the Sp17-2 gene variant from genomic DNA (Table 4.1). All primers were synthesized by Biosynthesis, Inc. (Lewisville, TX) and resuspended to 100pmol in sterile water.

The PCR products were prepared and analyzed as described in Chapter 1, section 3.3 with the following primer and template modifications: 200nM sense primer, 200nM anti-sense primer, 4uL cDNA or genomic DNA template. The PCR products were resolved by gel electrophoresis, visualized with UV light and photographed. As needed to visualize the amplified DNA fragments, a second round of PCR was performed as described above using 2uL of the first PCR cocktail as the template.

The appropriately sized PCR amplified DNA bands were excised, purified and subcloned into a TOPO vector as described in Chapter 1, section 3.6. The positive clones were purified and the DNA inserts were sequenced from the forward (M13R) and reverse (T7) directions using ABI BigDye Terminator chemistry (Applied Biosystems; Foster City, CA) by the DBS Automated DNA Sequencing Facility (University of California, Davis, CA). DNA sequence analysis, assembly and alignment was performed as described in Chapter 1, section 3.7.

3.4 Northern blot analysis

Northern blot analysis was performed using total RNA extracted from cultured cancer cell lines. The blots were prepared as described in Chapter 4, section 3.3. Briefly, a 100ug of total RNA samples were gel electrophoresed on a 1.5% formaldehyde gel. The gel was visualized with UV light and photographed. The RNA gel was transferred to Nylon paper (Micron Separations, Inc., Westborough, MA), air dried at room temperature, crosslinked with UV light and vacuum dried at 80^oC for 30 minutes.

The northern blot was pre-hybridized at 55^oC for 1 hour in hybridization solution and hybridized with a Sp17 gene specific, ^{32}P-labelled nick-translated (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) DNA probe (615bp; 10^6 cpm/mL) in hybridization solution at 55^oC overnight. The DNA probe was amplified from human

testis tissue by RT-PCR and spanned exon 3-5 and the 3'UTR of the Sp17 nucleotide sequence.

The blot was scanned with a STORM Imaging System (Molecular Dynamics, Sunnyvale, CA) and analyzed with Image Quant v1.2 computer software (Molecular Dynamics).

3.5 Western blot analysis

A western blot analysis was performed using total protein extracted from cultured cancer cell lines. Briefly, the confluent cancer cell lines were resuspended in TRIzol reagent. After extraction of total RNA (section 3.2), the total protein was extracted by sequential treatment with chloroform, isopropanol and 0.3M guanidine hydrochloride in 95% ethanol according to manufacturer guidelines. The total protein was resuspended in 1% SDS and stored at –20^{o}C.

The western blots were prepared as described in Chapter 4, section 3.4. The total protein extracts (100ug) were separated on a 12% SDS-PAGE polyacrylamide gel adjacent to a 230kda kaleidoscope pre-stained protein marker (BioRad Laboratories, Hercules, CA). The gels were transferred to nitrocellulose paper (Micron Separations, Inc., Westborough, MA) overnight.

The blot was blocked overnight with blocking reagent and the primary antibody (Chapter 3; anti-Sp17 or preimmune, 1:200) was incubated with the blot at room temperature for one hour. The blots were washed and the secondary antibody, HRPO goat anti-rabbit IgG (1:2000; Caltag, Burlingame, CA) was incubated with the blot at room temperature for one hour. The blots were washed and developed using chemiluminescent reagents (SuperSignal, Pierce).

4. Results

4.1 Sp17-1 gene transcript

The Sp17-1 transcript and the identification of alternative transcriptional start sites were examined in normal human and cancer cells by RT-PCR (primers Sg1a, Ssp1/2 and Ag1) (Figure 5.1A). The human adrenal, lymph node, muscle, ovary, spinal column and adult testes revealed a DNA fragment corresponding to the 849bp DNA band expected for Sp17-1a. Conversely, a 902bp DNA band corresponding to the Sp17-1b transcript was not observed in these tissues. Similarly, normal human esophagus, pmbcs, parathyroid and RA synovium exhibited both Sp17-1a and Sp17-1b transcript variants. However, a second round of PCR was needed to visualize the DNA bands from normal non-testes tissues, suggesting that the Sp17-1 transcript is in low abundance.

Conversely, the cancer cell lines, Hela S3, LuCaP, Du145, PC 3, 143B TK, A459 and CaLu1, exhibited DNA bands corresponding to both Sp17-1a and Sp17-1b transcripts (Figure 5.1B). The DNA fragments were visible after the first round of

PCR, suggesting that the Sp17-1 transcript is in relative abundance, as compared to normal human testes. All controls were negative for genomic DNA.

4.2 Sp17-2 gene

The Sp17-2 gene was examined in Hela S3 cancer cells by PCR (primer sets Sgr2 and ACDS, Shu and Ag2, Sgr2 and Ag2, Sg2full and ACDS and Shu and A2full). Unlike in normal tissues, PCR amplification of the Sp17-2 gene was not detected in reactions containing a Hela S3 genomic DNA template (Chapter 2). Conversely, RT-PCR analysis identified a Sp17-2 transcript in Hela S3 cDNA, free of genomic DNA (Figure 5.2AB).

4.3 Northern blot hybridization

Northern blots of total RNA extracted from multiple cancer cell lines were hybridized with a human Sp17 coding region specific ^{32}P-labelled nick-translated DNA probe. A strong Sp17 hybridization was detected to a single band of 950bp in the total mRNA extracted from all cancer cell lines, except the 4C8 Glioma cell line (Figure 5.3).

4.4 Western blot analysis

Western blot analysis of total protein extracted from multiple cancer cell lines was probed with a rabbit anti-recombinant Sp17 polyclonal antibody (Chapter 3). Anti-Sp17 reactivity was not observed in total protein extracted from any of the cancer cells examined (Figure 5.4). However, strong anti-Sp17 reactivity was observed against a single 18kDa band corresponding to recombinant Sp17 protein. In addition, western blots probed with rabbit pre-immune sera were negative for reactivity.

5. Discussion

The Sp17-1 nucleotide sequences was amplified from normal human tissues and cancer cell lines. RT-PCR analysis of the Sp17-1 alternative transcriptional start region in these tissues suggests that the Sp17-1a or Sp17-1b transcripts are tissue specific. For example, the Sp17-1a variant was detected in normal human adrenal, lymph node, muscle, ovary, spinal column and adult testes. Conversely, both the Sp17-1a and Sp17-1b transcriptional start sites were detected in normal human esophagus, pmbcs, parathyroid. Similarly, RA synovium and all cancer cell lines examined exhibited both transcriptional start sites. However, although this PCR was not designed to provide accurate quantitation, the shorter Sp17-1a transcript demonstrated a stronger signal that the Sp17-1b transcript. This difference suggests low level expression of the Sp17-1b transcript and a more dominant expression of the Sp17-1a transcript, as also observed in the cynomologous macaque and human testes (Chapter 1, Chapter 4).

The differential detection of the Sp17-1 transcript suggests a potentially pathogenic role for Sp17 depending on which transcriptional start site is expressed. However, whether the expression of one or both transcriptional start sites in the normal tissues reflects a predisposition to pathogenesis has yet to be fully investigated.

Additionally, the Sp17-2 gene was previously detected in the genomic DNA of the human and primate (Chapter 2). Interestingly, in cancer cell lines, a Sp17-2 transcript was amplified from a cDNA template, suggesting that Sp17-2 may be transcribed in this cancer cell line and potentially in other highly proliferating cells. Moreover, the detection of Sp17-2 in normal genomic DNA and neoplastic cDNA suggests that Sp17-2 may be a marker for highly proliferating cells. However, although Sp17-2 is not predicted to encode a full length Sp17 peptide, short Sp17-2 peptide fragments may be sufficiently immunogenic to target as a potential CT marker. In addition, Sp17 may also have an alternative role in highly proliferating and neoplastic cells, such as a mediator of signal transduction, protein synthesis and/or unregulated growth (17).

Sp17 mRNA expression was detected by northern blot analysis in basal cell carcinoma, lung cancer, prostate cancer, adenocarcinoma and osteocarcinoma cancer cell lines. However, Sp17 mRNA expression was not detected in normal human non-testes tissues. Thus, the detection of mRNA expression in testes and neoplastic cells, but not in non-testes tissues, strongly implicate Sp17 as a CT antigen.

The Sp17 protein was not detected in the cancer cell cultures, suggesting that the northern blots results may reflect Sp17-2 transcript rather than the Sp17-1 transcript. However, the Sp17 protein was detected in naturally occurring multiple myeloma cells, suggesting that the expression of the Sp17 protein may be limited to naturally occurring malignant tissues (14).

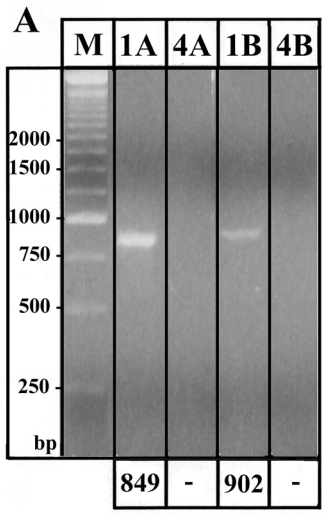
6. Conclusion

Sp17 transcription, mRNA expression and Sp17 protein translation was examined in multiple cancer cells as compared to human non-testes tissues. The differential detection of the Sp171a, Sp17-1b and Sp17-2 transcripts in cancer cell lines as compared to non-testes tissues, supports a potential pathogenic role for Sp17 in highly proliferating cells. Moreover, the detection of Sp17 mRNA expression in testes and neoplastic cell lines implicates Sp17 as a CT antigen. However, the regulation of Sp17 transcription and translation should be further investigated in naturally occurring neoplasias.

7. Tables and Figures

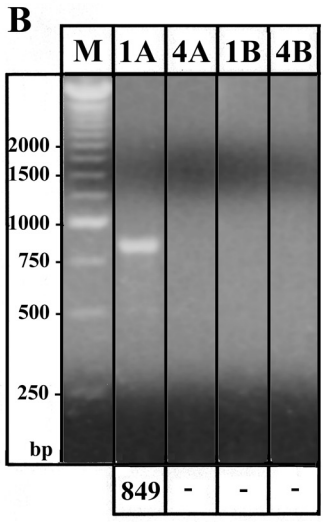
Table 5.1. . Sp17 specific sense and anti-sense primers used in RT-PCR to detect the Sp17-1 transcript, differentiate between alternative transcriptional start sites and detect the Sp17-2 gene variant.

CODING REGION			
Sense (Shu)	5'	CGCGGATCCATGTCGATTCCATTCTCC	3'
Anti-Sense (Ahu)	5'	CGGGGTACCAACCAGTGTCCTCACTTG	3'
GENE 1			
Sense (Sg1a)	5'	CGCCCCTTCTCGGCCGCCGTAG	3’
Sense (Ssp1/2)	5'	CCCAGCAACTAGAAAAACAACCGGAA	3’
Anti-Sense (Ag1)	5'	GATAATAGTTAGAACACATAACCTC	3'
GENE 2			
Sense (Sgr2)	5'	GAGGTCAGAGGAGGAAGTAAGCTG	3'
Sense (Sg2)	5'	CCCTAGAACATGAGGTCAGAGGAG	3'
Sense (Shu)	5'	CGCGGATCCATGTCGATTCCATTCTCC	3'
Sense (Sg2full)	5'	CCAATGACTGATGGACACAGACTG	3'
Anti-Sense (aCDS)	5'	GAGTCTAAGATGGTGACTGATG	3'
Anti-Sense (Ag2)	5'	CCATGAGCCAAAATGGTGGTAGTC	3'
Anti-Sense (Ag2full)	5'	GAGATGTGATAGCTGGCACTCATG	3'



HUMAN				
Tissue	1A	4A	1B	4B
Esophagus	+	-	+	-
Parathyroid	+	-	+	-
PMBC	+	-	+	-
Synovium	+	-	+	-
	849	-	902	-

CANCER				
Cell	1A	4A	1B	4B
Lu CaP	+	-	+	-
Du 145	+	-	+	-
PC3	+	-	+	-
143 BTK	+	-	+	-
Hela S3	+	-	+	-
A549 P	+	-	+	-
Calu 1	+	-	+	-
	849	-	902	-



HUMAN				
Tissue	1A	4A	1B	4B
Adrenal	-	-	+	-
Lymph node	-	-	+	-
Skeletal muscle	-	-	+	-
Ovary	-	-	+	-
Spinal Column	-	-	+	-
Testes	-	-	+	-
	849	-	902	-

Figure 5.1. RT-PCR analysis of the Sp17-1 transcript in multiple human tissues and cancer cell lines. Sp17 specific primers were used to differentiate between alternative transcriptional start sites, Sp17-1a (1A; 849bp) and Sp17-1b (1B; 902bp). The DNA fragments were measured by a DNA marker (M) in bp. The PCR cocktails were prepared with the following template 1: cDNA; 4: none (Chapter 1, Table 1.1). **A.** RT-PCR analysis revealed DNA amplification of the both Sp17-1 variants, Sp17-1a and Sp17-1b, in the tissues listed on the right. **B.** PCR analysis revealed DNA amplification of Sp17-1a in the tissues listed on the right. All controls (4A, 4B) were negative for genomic DNA.

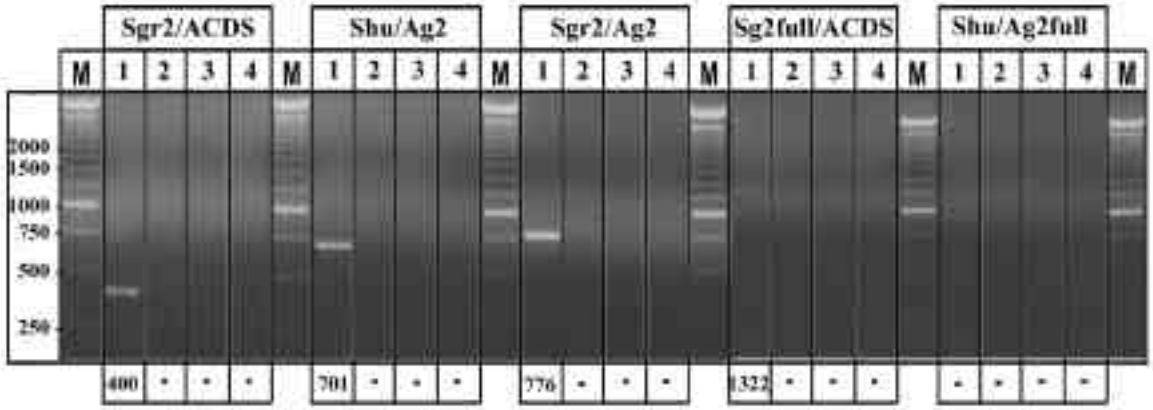


Figure 5.2. PCR analysis of the Sp17-2 gene in Hela S3 cancer cell lines. Sp17-2 specific primers were used to elucidate Sp17-2 in the cDNA versus the genomic DNA. The PCR cocktails were prepared with the following templates 1: cDNA; 2: gDNA; 3: RNA; 4: none (Chapter 1, Table 1.1). The PCR products were measured in bp adjacent to a DNA marker (M). All controls (3 and 4) were negative for genomic DNA.

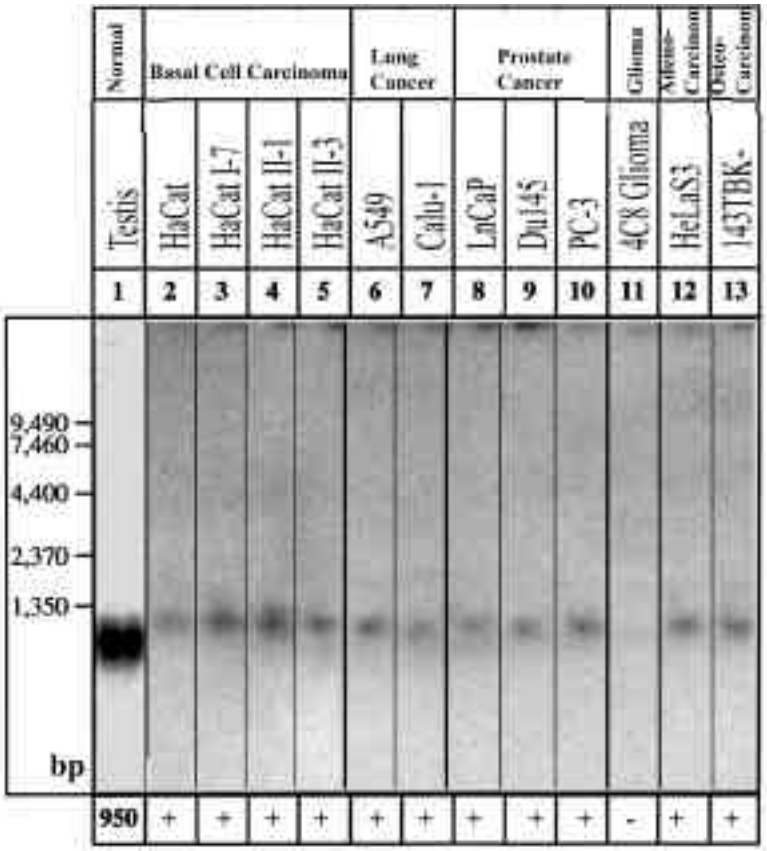


Figure 5.3. Northern blot analysis of total RNA extracted from multiple cancer cell lines and normal adult cynomologous macaque testis RNA (positive control). The blot was hybridized with a ^{32}P-labeled nick-translated Sp17 DNA probe. Strong hybridization to the cancer RNAs was detected at ~950bp as measured by a RNA marker. However, hybridization was not observed to 4C8 Glioma RNA.

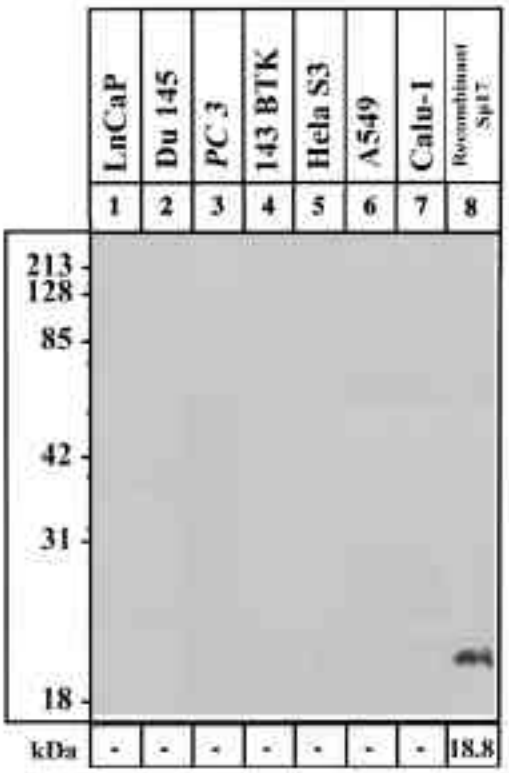


Figure 5.4. Western blot analysis of total protein extracted from multiple cancer cell lines. The blot was probed with a rabbit anti-recombinant Sp17 sera. Strong hybridization to recombinant Sp17 protein (18.8kDa) was observed. However, anti-Sp17 reactivity was not detected to any cancer cell lines tested.

8. References

1 Chen Y. T. and L. J. Old. 1999. Cancer-testis antigens: targets for cancer immunotherapy. Cancer J Sci Am. 5:16-7.

2 Chen Y. T., A. O. Gure, S. Tsang, E. Stockert, E. Jager, A. Knuth and L. J. Old. 1998. Identification of multiple cancer/testis antigens by allogeneic antibody screening of a melanoma cell line library. Proc. Natl. Acad. Sci. 95:6919-6923.

3 Sahin U, M. Koslowski, O. Tureci, T. Eberle, C. Zwick, B. Romeike, J. R. Moringlane, K. Schwechheimer, W. Feiden, M. Pfreundschuh. 2000. Expression of cancer testis genes in human brain tumors. Clin Cancer Res. 6:3916-22.

4 Chen C, G. Chen, H. Lee, G. Huang, P. Yang, L. Tsai, D. Chen and J. Sheu. 2001. Expressions of cancer-testis antigens in human hepatocellular carcinomas. Cancer Lett. 164:189-195.

5 Scanlan M. J., N. K. Altorki, A. O. Gure, B. Williamson, A. Jungbluth, Y. T. Chen and L. J. Old. 2000. Expression of cancer-testis antigens in lung cancer: definition of bromodomain testis-specific gene (BRDT) as a new CT gene, CT9. Cancer Lett. 150:155-64.

6 Stockert E, E. Jager, Y. T. Chen, M. J. Scanlan, I. Gout, J. Karbach, M. Arand, A. Knuth and L. J. Old. 1998. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. J Exp Med. 187:1349-54.

7 O'Rand, M. G., E. E. Widgren and S. J. Fisher. 1988. Characterization of the rabbit sperm membrant autoantigen, RSA, as a lectin-like zona binding protein. Dev. Bio. 129:231-240.

8 Richardson, R. T., N. Yamasaki and M. G. O'Rand. 1994. Sequence of a rabbit sperm zona pellucida binding protein and localization during the acrosome reaction. Dev. Biol. 165:688-701.

9 Adoyo, P. A., I. A. Lea, R. T. Richardson, E. E. Widgren and M. G. O'Rand. 1997. Sequence and characterization of the sperm protein Sp17 from the baboon. Mol. Reprod. Dev. 47:66-71.

10 Lea I. A., R. T. Richardson, E. E. Widgren and M. G. O'Rand. 1996. Cloning and sequencing of cDNAs encoding the human sperm protein, Sp17. Biochim Biophys Acta. 1307:263-6.

11 Tatlow, D., R. Brownlie, L. A. Babiuk and P. Greibel. 2000. Differnetial display analysis of gene expression during induction of mucosal immunity. Immunogenetics. 52:73-80.

12 Takeoka, Y., T. P. Kenny, M. E. Gershwin, H. Yago and D. L. Robbins. Sperm protein 17 gene expression in rheumatoid arthritis synoviocytes. (Submitted).

13 Dong, G., E. Loukinova, C. W. Smith, Z. Chen and C. Van Waes. 1997. Genes differentially expressed with malignant transformation and metastatic tumor progression of murine squamous cell carcinoma. J. Cell. Biochem. Suppl. 28-29:90-100.

14 Lim S. H., Z. Wang, M. Chiriva-Internati and Y. Xue. 2001. Sperm protein 17 is a novel cancer-testis antigen in multiple myeloma. Blood. 97:1508-1510.

15 Seuningen I. V., M. Perrais, P. Pigny, N. Porchet, J. P. Aubert. 2000. Sequence of the 5'-flanking region and promoter activity of the human mucin gene MUC5B in different phenotypes of colon cancer cells. Biochem J. 348:675-686.

16 Chen, S., R. S. Maul, H. R. Kim and D. D. Chang. 2000. Characterization of the human EPLIN (Epithelial protein lost in neoplasm) gene reveals distinct promoters for the two EPLIN isoforms. Gene. 248:69-76.

17 O'Rand, M. G. and E. E. Widgren. 1994. Identification of sperm antigen targets for immunocontraception: B-cell epitope analysis of Sp17. Reprod. Fertil. Dev. 6:289-296.