Haploinsufficiency and Reduced Expression of Genes Localized to the 8p Chromosomal Region in Human Prostate Tumors

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Cytogenetic and molecular studies have suggested that deletion or rearrangement of sequences that map to the short arm of chromosome 8 may be permissive for tumorigenesis in several organ systems, and in human prostate tumors in particular. In this study, we hypothesized that genes deleted for one copy and localized to the 8p chromosomal region may be transcriptionally down-regulated or ablated in affected human prostate tumor tissues. To test this hypothesis, we used cDNA microarray analysis to determine the transcriptional profiles for 259 transcribed sequences mapping to the 8p chromosomal region for seven human prostate tumor xenografts, completely characterized for numerical and structural alterations on chromosome 8, and five normal human prostate tissues. These experiments identified 33 genes differentially expressed between normal and malignant prostate tissues, the majority of which (28/33, 85%) were transcriptionally down-regulated in malignant compared to normal human prostate tissues. These findings, that haploinsufficiency and transcriptional down-regulation for genes mapping to 8p are largely coincident in human prostate tumors, should provide a powerful tool for the identification of tumor-suppressor genes associated with human prostate cancer initiation and progression.

INTRODUCTION

Cytogenetic and molecular studies have suggested that deletion or rearrangement of sequences that map to the short arm of chromosome 8 may be permissive for tumorigenesis in several organ systems. In prostate carcinoma, early work by Bergerheim et al. (1993) by the use of Southern blot techniques showed high frequencies of deletion of distal 8p sequences in human prostate tumors, and suggested that a breakpoint existed between the *NEFL* locus at 8p21 and the *PLAT* locus at 8p21–8p11. Subsequent work in our laboratory identified a major region of deletion mapping to 8p22 in human prostate cancer (Macoska et al., 1995). This was corroborated by the description and later characterization of a homozygous deletion within the 8p22–p23 region in a prostate tumor (Bova et al., 1993, 1996). The use of polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) techniques in our laboratory and by others confirmed frequent loss of distal 8p sequences and showed that this region of deletion could be quite extensive, including the entire 8p chromosome arm or large portions of 8p extending from 8pter to 8p11–12 (MacGrogan et al., 1994; Macoska et al., 1994; Trapman et al., 1994; Prasad et al., 1998). Two large studies from our laboratory showed interstitial deletions of distal sequences mapping to 8p22, 8p21, and 8p11–p12, including homozygous deletion of sequences mapping to the *D8S87* locus at 8p12, in human prostate tumors (Macoska et al., 1995; Prasad et al., 1998). As a result of these studies, we and others have hypothesized that one or more tumor-suppressor genes critical for prostate tumorigenesis map to 8p.

It is reasonable to propose that the transcription of genes mapping to chromosomal regions deleted

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for one or both copies may be down-regulated or ablated in the affected tumor tissues. If so, then deletion-induced haploinsufficiency alone may result in reduced or ablated gene transcription in affected tumor tissues. To test this hypothesis, we determined the RNA profiles of seven human prostate tumor xenografts, completely characterized for numerical and structural alterations on chromosome 8, and five normal human prostate tissues for 677 transcribed sequences mapping to chromosome 8, by use of cDNA microarray analysis. These experiments demonstrate the down-regulation or ablation of transcripts from 28 genes mapping to regions of 8p deleted in the prostate tumor xenografts and are consistent with the identification of these sequences as candidate tumor-suppressor genes that are inactivated during prostate tumorigenesis.

MATERIALS AND METHODS

Tissue Acquisition and Characterization

Normal benign human prostate tissue was obtained after radical prostatectomy from patients diagnosed with prostate cancer. After an initial pathologic evaluation of radical prostatectomy tissue, presumed normal tissue was snap-frozen in liquid nitrogen and stored at −70°C. One section from each specimen was examined after staining with H&E, and the specimen was included in the study if the section demonstrated at least 70% epithelial cells. All radical prostatectomy specimens were acquired with full Institutional Review Board approval. The LuCaP series of human prostate tumor xenografts was chosen for these studies because it constitutes a renewable, largely characterized source of human prostate tumor tissue (Ellis et al., 1996; Williams et al., 1997; Linja et al., 2001; Corey et al., 2002; Laitinen et al., 2002; True et al., 2002). The xenografts were maintained by serial passage in intact athymic Balbc nu/nu male mice and were harvested at passage 45 for LuCaP 23.1, 40 for LuCaP 23.8, 25 for LuCap 23.12, 67 for LuCaP 35, 17 for LuCaP 41, 22 for LuCaP 49, and 19 for LuCaP 58. At sacrifice, tumor samples were harvested and frozen in optimum cutting temperature medium.

Alleleotyping

PCR was used for amplification of sequences containing highly polymorphic microsatellite repeat markers at loci of interest on chromosome 8 (12 sequences). The cytogenetic and map position for each locus was obtained from the UCSC Genome Bioinformatics database (http://genome.cse.ucsc.edu/). Primer sequences were obtained from public databases maintained by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), as accessed through the Internet. DNA was prepared from frozen xenograft tissues, and PCR reactions were performed as previously described (Prasad et al., 1998). Allelic dosage was scored as "1" if a single band was observed in the PCR reaction or as "2" if two discrete bands were observed.

RNA Profiling by cDNA Microarray

RNA was prepared from normal benign prostatic and xenograft tissues after homogenization and lysis in Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's instructions. The RNA was further purified by use of RNeasy reagents (Qiagen, Valencia, CA), and 20 μg of total RNA from each sample was labeled with Cy5 fluorescent dye by use of a CyScribe first-strand cDNA labeling kit. For each experiment, 20 μg of Universal Human Reference RNA (Stratagene, La Jolla, CA) was labeled with Cy3 by use of similar protocols. After purification on AutoSeq G50 columns (Amersham Biosciences, Piscataway, NJ), the labeled probes were combined, dried down to 5 μl, and added to 45 μl SlideHyb #1 hybridization buffer (Ambion, Houston, TX), prewarmed to 68°C. The probe/hybridization mixture was pre-incubated at 68°C for 5 min, then transferred to the cDNA microarray slide, which was coverslipped and incubated in a moist chamber at 45°C for 16 hr. The slides were washed for 10 min each in 2× SSC, 0.2% SDS at 45°C, 2× SSC at room temperature, and 0.2× SSC at room temperature; rinsed; dried; and evaluated with a GenePix 4000A Axon laser scanner and associated software (Axon Instruments, Burlingame, CA).

Chromosome 8 cDNA Array

The custom chromosome 8 array was produced at the University of Michigan Comprehensive Cancer Center cDNA and Affymetrix Microarray Core. This array consisted of PCR products amplified from 677 Research Genetics (Huntsville, AL) human cDNA clones whose sequences mapped to chromosome 8. To identify these clones, our laboratory annotated the GenBank accession numbers associated with all 25,921 Research Genetics human cDNA clones with chromosomal location by use of information from the National Center for Biotechnology Information Ensembl database (http://www.ensembl.org/homo_sapiens) and the

UCSC Genome Bioinformatics database (http://genome.cse.ucsc.edu/). According to information in these databases, 677 clones contained sequences mapping to human chromosome 8; 259 mapped to 8p and 418 mapped to 8q. A complete list of cDNAs spotted onto the chromosome 8 array can be obtained at http://www-personal.umich.edu/~jcoska/Chrom8Array.xls.

Northern Blot Analysis

A 20-μg sample of total RNA from each specimen isolated as described above was resolved on 1% formaldehyde agarose gels and transferred to a Nytran membrane by use of a TurboBlotter apparatus (Schleicher & Schuell, Keene, NH). Each blot was probed with radiolabeled PCR products amplified for the gene of interest, hybridized by use of the ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA), and visualized on X-OMAT film (Kodak, Rochester, NY).

Statistical Analysis

Data used for this study were background-subtracted median intensity values with average log intensities greater than 8. Data from each array were normalized to remove dye-bias by use of a rank-invariant normalization method (Tseng et al., 2001). This normalization method attempts to find genes that do not appear to be differentially expressed in the two samples hybridized to a microarray. A non-linear regression (lowess) line was then fitted to these points and used for normalizing the data. Before making comparisons between arrays, we performed a quantile normalization of the rank-invariant normalized array data. We assumed that the distribution of the array data was the same for all arrays, but that the scale of the data may be quite different. To correct for this difference in scale, we used data from one array as baseline and adjusted all other arrays to match (Shedden, 2002a,b). Differentially expressed genes were detected with SAM (significance analysis of microarrays) (Tusher et al., 2001). Hierarchical clustering was used for grouping of genes with similar expression patterns (Eisen et al., 1998).

RESULTS

Allelotyping

Allelotyping experiments showed that LuCaPs 23.1, 49, and 58 demonstrated one allele for all twelve 8p loci examined, suggesting that one copy of the entire short arm of chromosome 8 was deleted in those specimens. LuCaP 23.8 also demonstrated extensive deletion for one copy of 8p, although the results were inconclusive for the most proximal locus, *D8S255*, thus precluding an assessment of complete deletion. LuCaP 23.12 demonstrated one allele for 10 loci extending from *D8S504* to *D8S87* and two alleles at locus *D8S1121*, suggesting that the most proximal portion of 8p was retained in that xenograft. LuCaPs 35 (loci *D8S504-NEFL*) and 41 (loci *D8S504-D8S540*) demonstrated deletion for one copy of distal 8p with retention of two copies of proximal 8p sequences (Table 1). Taken together, the allelotyping data demonstrated deletion for one copy of the entire 8p chromosomal region for all xenografts except LuCaPs 35 and 41, which were deleted for one copy of the distal 8p region only. These results were consistent with those obtained by others who used FISH and comparative genome hybridization (CGH), except that LuCaP 35 evinced loss of distal 8p by allelotyping but not by CGH analysis, and LuCaP 41 demonstrated loss of all of one copy of the entire 8p arm by CGH analysis but not by allelotyping (Williams et al., 1997; Laitinen et al., 2002). In addition, FISH and CGH analyses demonstrated gain of one copy of 8q in LuCaPs 23.8, 23.12, 35, and 58, and amplification of 8cen-q21 and 8q24-qter in LuCap 23.8, 8q21–q22 in LuCaP 35, and 8q23-qter in LuCaP 58 (Williams et al., 1997; Laitinen et al., 2002).

cDNA Microarray Analysis

The xenograft tissues were transcriptionally profiled for coding sequences mapping to chromosome 8. To accomplish this, we constructed a chromosome 8 array by use of PCR products amplified from 677 clones from the Research Genetics human cDNA library whose sequences mapped to chromosome 8. The chromosome 8 sequence specificity and the exact physical location of each transcript on chromosome 8 were established by annotation of the cloned sequences with chromosome mapping information derived from the National Center for Biotechnology Information Ensembl database (http://www.ensembl.org/homo_sapiens) and the UCSC Genome Bioinformatics database (http://genome.cse.ucsc.edu/).

Five specimens of histologically verified normal benign human prostate tissues obtained after radical prostatectomy from patients diagnosed with prostate cancer were used as controls. The five normal benign prostate tissues and LuCaP tumors 23.1, 23.12, 23.8, 35, 41, 49, and 58 were analyzed for the gene expression pattern of tran-

TABLE 1. Allelotyping of LuCaP Xenografts for 8p Sequence Dosage

Cyto band	p23.3	p23.1	p22	p22	p21.2	p12	p12	p12	p12	p12	p11.21
Locus	D8S504	D8S277	D8S549	D8S261	NEFL	D8S540	D8S513	D8S535	D8S505	D8S87	D8S1121	D8S255
Base Position	825022	7046339	14664896	17799405	23778854	28591532	32088853	32110141	35378304	36228896	36729210	40483607
LuCaP 23.12	—	—	—	—	—	—	—	—	—	—	2	—
LuCaP 23.8	—	—	—	—	—	—	—	—	—	—	—	
LuCaP 49	—	—	—	—	—	—	—	—	—	—	—	—
LuCaP 58	—	—	—	—	—	—	—	—	—	—	—	—
LuCaP 23.1	—	—	—	—	—	—	—	—	—	—	—	—
LuCaP 35	—	—	—	—	—	2	—	2	—	—	2	2
LuCaP 41	—	—	—	—	—	—	2	—	2	2	—	—

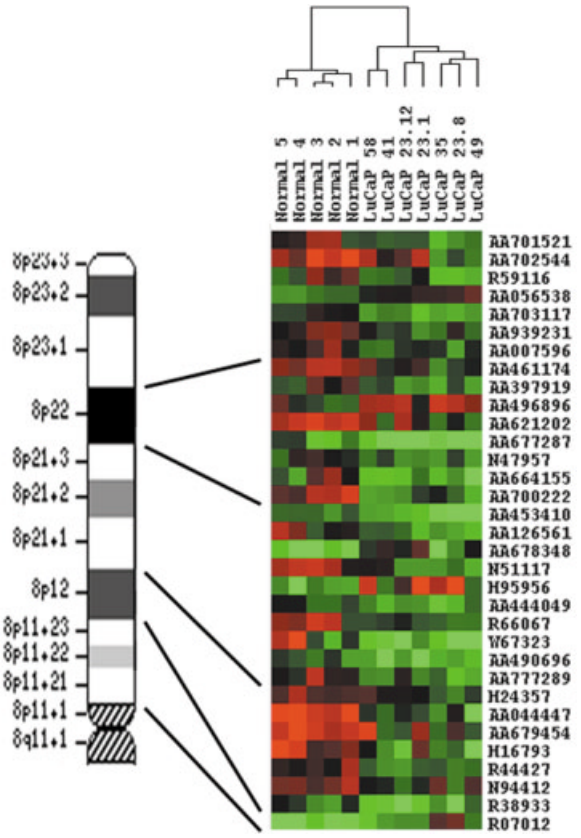

Figure 1. Hierarchical clustering of differentially expressed genes mapping to 8p. Normal human prostate tissues cluster together on the left; malignant human prostate tissues cluster together on the right of the diagram. Genes are identified by GenBank accession number and are arranged by chromosomal position, from 8pter (**top**) to 8p11 (**bottom**). Green boxes indicate statistically significant transcriptional down-regulation in tumors compared to normal specimens; red indicates up-regulation, and black indicates that expression was not statistically significant for that specimen; 28/33 differentially expressed genes are down-regulated in the tumor specimens compared to the normal prostate specimens.

scripts mapping to chromosome 8 by use of a custom chromosome 8 array. Because the intent in these studies was to determine whether a common set of genes were transcriptionally down-regulated in tissues deleted for one copy of 8p, the xenograft data were considered together rather than separately. After background subtraction and quantile normalization of the rank-invariant normalized array data, differentially expressed genes were detected by use of SAM (Tusher et al., 2001; Shedden, 2002a,b).

Hierarchical clustering was used to group tissues and genes with similar expression patterns (Eisen et al., 1998). As expected, the five normal tissues clustered together, as did the seven xenograft tissues (Fig. 1). Of the three xenografts originally derived from three separate metastatic deposits

from the same patient (LuCaPs 23.1, 23.8, and 23.12), LuCaPs 23.1 and 23.12 clustered together, whereas LuCaP 23.8 clustered with LuCaP 35. Interestingly, LuCaPs 23.1 and 23.12 were both characterized by gain of the entire 8q chromosomal arm, whereas LuCaPs 23.8 and 35 were distinguished by gain of 8q and amplification of discrete regions on 8q (Williams et al., 1997; Laitinen et al., 2002).

Statistical analysis demonstrated that the mean expression of 88/677 (13%) genes spotted onto the array was statistically significantly different in prostate tumor xenografts compared to normal prostate tissues. Of the 88 differentially expressed genes, 33 mapped to 8p and 55 to 8q. Twenty-eight of 33 (85%) of the differentially expressed genes that mapped to 8p were down-regulated in the prostate tumor xenografts compared to normal prostate tissues. These results are consistent with the expectation that a majority of genes mapping to 8p would be transcriptionally down-regulated in the prostate tumor xenografts, which are deleted for one copy of 8p. The five gene sequences that constitute the exceptions to this trend encode two ESTs (GenBank H95956, R07012), two hypothetical proteins (GenBank AA056538, AA678348), and the *ATIP1* gene (GenBank AA496896). Interestingly, three genes previously identified as tumor-suppressor genes mapping to 8p (*NKX3.1*, *LZTS1*, and MSR1) were transcriptionally down-regulated in a subset of the 8p-deleted xenografts but did not achieve significance by SAM analysis. *NKX3.1* was down-regulated in LuCaPs 35 and 49, MSR1 was down-regulated in LuCaPs 41 and 49, and *LZTS1* was down-regulated in LuCaPs 23.1, 23.12, 49, and 58.

The expression pattern on 8q was more complex, with 23 genes (42%) up-regulated and 32 genes (58%) down-regulated in the prostate tumor xenografts compared to normal prostate tissues. There were no obvious correlations between gene expression level and 8q copy number in the prostate tumor xenografts, although the proportion of up-regulated genes was significantly higher on the q arm than on the p arm ($P < 0.017$). The identities of the differentially expressed genes mapping to 8p are listed in Table 2. The observation of transcriptional down-regulation for the majority of differentially expressed genes mapping to 8p in the xenografts compared to normal prostate tissues is consistent with the allelotyping data demonstrating loss of all or part of one copy of 8p in the seven xenografts (Table 1 and Fig. 1).

Validation of cDNA Microarray Data

We conducted Northern blot analysis to determine whether the gene expression profiles acquired from the cDNA microarray data were accurate. Two transcripts originating from genes mapping to the proximal 8p region were chosen for the validation studies, *RBP-MS* and *TEM5*. *RBP-MS* was chosen in particular because PCR products from two different cDNA clones representing this sequence were spotted onto the array, and expression data acquired from both spots were consistent with transcriptional down-regulation of *RBP-MS* in the xenografts (Table 2). The same Northern blot filter was sequentially hybridized to radiolabeled probes for *RBP-MS*, *TEM5*, and *GAPDH* (as a loading control) and demonstrated expression of all three genes in three of the same normal human prostate tissues previously analyzed by cDNA microarray (N4, N5, and N6) (Fig. 2). However, both *RBP-MS* and *TEM5* were not expressed in LuCaPs 49, 23.1, and 41, whereas *GAPDH* was abundantly expressed in all three prostate tumor xenograft tissues (Fig. 2). These results were consistent with the cDNA microarray results and demonstrated ablation of *TEM5* and *RBP-MS* gene expression in prostate tumor xenografts deleted for one copy of 8p.

DISCUSSION

These studies were intended to determine whether gene dosage correlated with gene expression level in human prostate tumors with known structural and numerical alterations involving chromosome 8. The xenograft tissues were particularly suited to these studies because they provided a renewable source of prostate tumor tissue with known 8p deletions that could be extensively characterized by RNA profiling. Prostate cancer cell lines could also have been examined, but proved to be unsuitable because they were far less "uniform" in their chromosome 8 composition, and it was unclear whether some cell lines demonstrated deletion in addition to translocation of 8p sequences. For example, LNCaP cells have four copies of chromosome 8; DU145 exhibits a der(7;8)(p10;q10) translocation; and PC3 exhibits del(8)p21, der(8) t(X;8)(q10;q10), and der(8)t(8;15)(q10;10) chromosomes (Beheshti et al., 2000). The 22Rv1 cell line actually demonstrates gain for a portion of 8p, including 8p12–p22 (Laitinen et al., 2002). Therefore, we chose to profile the LuCaP series of human prostate tumor xenografts with known 8p

TABLE 2. Differential Expression of 8p Transcripts

Symbol	GenBank accession no.	Fold change	Base position	Trend	Cyto band
ESTs	AA701521	−2.488	131657	Down	8p23
KIAA0711	AA702544	−1.912	1633119	Down	8p23
ESTs	R59116	−1.948	2434358	Down	8p23
FLJ11210	AA056538	3.402	7055704	Up	8p23
DKFZp434D2426	AA703117	−3.237	9116035	Down	8p23
TNKS	AA939231	−2.135	9721956	Down	8p23
ESTs	AA007596	−1.977	12496945	Down	8p23
ZDHHC2	AA461174	−2.511	17416305	Down	8p22
FLJ32642	AA397919	−1.901	17488997	Down	8p22
ATIP1	AA496896	2.580	17837267	Up	8p22
ESTs	AA621202	−2.644	17846673	Down	8p22
FGL1	AA677287	−2.785	18045902	Down	8p22
PCM1	N47957	−1.943	18132400	Down	8p22
ASAH1	AA664155	−2.384	18234223	Down	8p22
SCAM-1	AA700222	−4.063	22724596	Down	8p22
TNFRSF10B	AA453410	−2.201	23264878	Down	8p21
STC1	AA126561	−2.717	24085387	Down	8p21
FLJ25804	AA678348	2.532	25792008	Up	8p21
ESTs	N51117	−3.448	27265764	Down	8p21
ESTs	H95956	2.756	29620387	Up	8p21
DUSP4	AA444049	−2.003	29960552	Down	8p21
RBPMS	R66067	−2.823	30220588	Down	8p21
RBPMS	W67323	−2.871	30870731	Down	8p21
PPP2CB	AA490696	−2.129	31553844	Down	8p21
GSR	AA777289	−2.333	31641629	Down	8p21
NRG1	H24357	−1.848	33227982	Down	8p12
TEM5	AA044447	−3.954	36112103	Down	8p12
STAR	AA679454	−2.335	38871582	Down	8p12
C8orf4	H16793	−2.726	40119560	Down	8p12
POLB	R44427	−2.926	42052597	Down	8p12
IKBKB	N94412	−1.969	42092090	Down	8p12
PLAT	R38933	−2.076	42273247	Down	8p12
ESTs	R07012	2.481	48374593	Up	8p11

deletions and five normal human prostate tissues diploid for 8p as controls.

In this study, we hypothesized that gene expression levels would be reduced or ablated in tumors deleted, or haploinsufficient, for one copy of 8p. Consistent with this hypothesis, 28/33 (85%) of the differentially expressed genes that mapped to 8p were down-regulated in the prostate tumor xenografts compared to normal prostate tissues.

Two of the genes identified as transcriptionally down-regulated in xenografts deleted for 8p were *TEM5* and *RBP-MS*. *TEM5* maps to 8p12 and encodes a seven-pass transmembrane protein (Carson-Walter et al., 2001). Although the protein was originally described as exclusively expressed on the surface of tumor endothelial cells, our studies show that the *TEM5* gene transcript was clearly expressed in normal, but not malignant, prostate epithelium. *RBP-MS* maps to 8p21 at the distal boundary of the 8p21–8p12 deletion breakpoint previously observed in human prostate tumors (Shimamoto et al., 1996; Prasad et al., 1998). The *RBP-MS* gene encodes an RNA-binding protein with complex alternative splicing patterns, resulting in at least 12 different transcripts (Shimamoto et al., 1996). The original description of this gene also showed strong expression of *RBP-MS* transcripts in the human prostate, as well as the heart, small and large intestines, and ovaries (Shimamoto et al., 1996). The more conserved N-terminal region contains RNA-binding sequences homologous to similar motifs in the *Drosophila couch potato* gene transcript, whereas the COOH-terminal region is highly divergent between the splice variants. Recent studies demonstrated that *couch potato* is involved in peripheral nervous system development, but is also highly expressed in the ring gland, the major *Drosophila* endocrine organ, throughout development (Harvie et al., 1998). The presence of RNA-binding motifs suggests that the RBP-MS

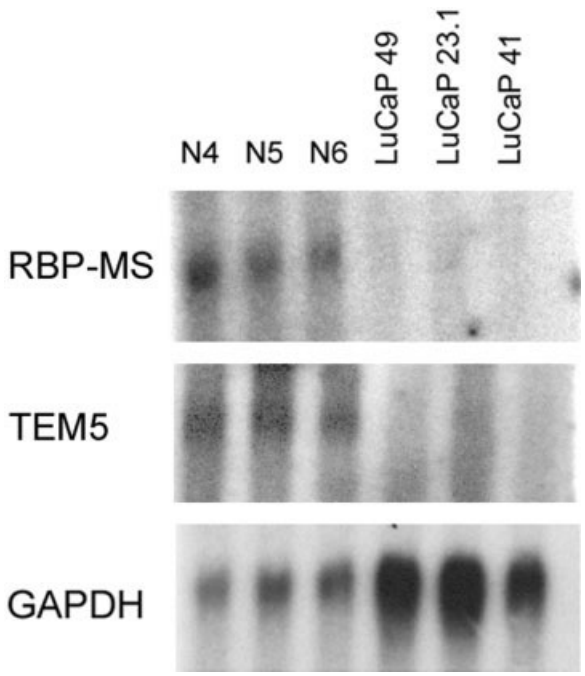

Figure 2. Northern blot validation of cDNA microarray data. Total RNA from normal specimens N4, N5, and N6 and from tumor specimens LuCaP 49, 23.1, and 41 were electrophoresed and blotted as described in the text. The Northern blot filter was sequentially hybridized to radiolabeled probes for *RBP-MS*, *TEM5*, and *GAPDH* (as a loading control). The normal prostate specimens demonstrated abundant expression of all three genes. However, *RBP-MS* and *TEM5* were not expressed in LuCaPs 49, 23.1, and 41, whereas *GAPDH* was clearly expressed in all three prostate tumor xenograft tissues. These results are consistent with the cDNA microarray results and demonstrate ablation of *TEM5* and *RBP-MS* gene expression in prostate tumor xenografts deleted for one copy of 8p.

protein may be involved in RNA processing, transport, stabilization, or translation. Moreover, its high expression levels in the major endocrine organs of Drosophila (ring gland) and humans (ovaries, prostate) suggests that the RBP-MS protein may play a major role in these processes during sexual maturity and/or reproduction.

Several recent studies have suggested that haploinsufficiency for specific genes is associated with the initiation or progression of sporadic tumors in mice and humans. In particular, haploinsufficiency attributed to chromosomal deletion rather than base sequence mutation associated with tumorigenesis and progression has been described for the *PTEN* gene in mouse prostate tumors, the *p27KIP1* gene in human acute lymphocytic leukemia, and the *KLF5* transcription factor gene in human breast tumors (Komuro et al., 1999; Kwabi-Addo et al., 2001; Chen et al., 2002). Taken together, these data suggest that the transcriptional down-regulation of genes associated with deletion-induced haploinsufficiency may help identify candidate tumor-suppressor genes in human cancers. In particular, our finding that haploinsufficiency and transcriptional down-regulation for genes mapping to 8p are largely coincident in human prostate tumors provides a powerful tool for the identification of tumor-suppressor genes associated with human prostate cancer initiation and progression.

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