Ribosomal Protein L19 Is a Prognostic Marker for Human Prostate Cancer
Alix Bee,1 Youqian Ke,1 Shiva Forootan,1 Ke Lin,2 Carol Beesley,1 Sharon E. Forrest,3 and Christopher S. Foster1

Abstract Microquantity differential display analysis of gene expression profiles between benign (PNT2) and malignant (PC3M) human prostate cell lines identified the gene encoding ribosomal protein L19 (RPL19) to be overexpressed in the malignant cells. Northern blot hybridization analysis done on a wide range of human cell lines and tissues confirmed the level of RPL19 mRNA to be 5-fold higher in malignant cell lines and 8-fold higher in malignant tissues, when compared with their benign counterparts. Analysis of RPL19 mRNA expression by in situ hybridization revealed a significant increase of RPL19 expression in a substantial number of prostate cancers. All of the eight normal prostatic tissues were unstained (100%). Of 32 benign prostatic hyperplasia (BPH) tissues, 15 (46.9%) were unstained, 9 (28.1%) stained weakly, and 8 (25%) stained moderately. Among 87 carcinomas, only 7 (8.1%) were unstained, whereas 22 (25.2%) stained weakly, 21 (24.1%) stained moderately, and 37 (42.61%) stained strongly. The intensity of staining of the malignant specimens was significantly higher than that of normal and BPH specimens ($\chi^2: n = 127, P < 0.001$). Gleason scores of the carcinomas correlated with RPL19 expression ($\chi^2: n = 87, P < 0.001$). Kaplan-Meier survival analysis confirmed increased RPL19 expression to be highly predictive of shorter patient survival ($P < 0.05$), revealing RPL19 to be a sensitive predictor of prostate cancer progression. Expression of this protein could be a valuable marker in prostate cancer diagnosis and patient management.

Effective diagnosis and treatment of prostate cancer is compromised by the limitations of unreliable prognostic markers for the disease. To date, the preferred tumor marker for prostate cancer is prostate-specific antigen (PSA). However, PSA is not prostate cancer specific because elevated levels may be caused by benign prostatic hyperplasia (BPH) or prostatitis. Only with rising levels does the positive predictive value of PSA become more valid (1). Men with reference ranges of PSA (<4.0 ng/mL) may have prostate cancer, but only when PSA levels exceed 10 ng/mL does the test become more accurate. Although PSA screening has enabled diagnosis of the disease at an earlier stage, most patients still have moderately or poorly differentiated tumors at diagnosis. Thus, there is a need to identify new markers that are more sensitive during early stages of prostate cancer development. Recent discovery of novel genes associated with the malignant progression of prostate cancer (2–6) has provided some potential candidates as more reliable biomarkers to predict the status of the disease.

Prostate cancer progression involves complex mechanisms determined by differential gene expression within evolving malignant cells, including genes recently identified and characterized (2, 3, 7). Microquantity differential display, an improved approach (7) to the original method designed by Liang and Pardee (8), identified the gene encoding for ribosomal protein L19 (RPL19) to be differentially expressed in human malignant prostate epithelial cell lines, with significant enhancement of gene expression occurring in the malignancies. RPL19 is a member of a family comprising >70 different proteins that form the large and small ribosomal subunits. RPL19 has not been previously implicated in the progression of prostate cancer nor has its prognostic value been investigated. However, differential expression of RPL19 has been identified in other epithelial malignancies. For example, increased levels are found at the core of malignant melanomas when compared with levels expressed at the tumor margin (9), and the gene has been highlighted as a potential biomarker of breast cancer (10). Therefore, the potential of RPL19 as a prognostic indicator may be of use not only in prostate cancer but also in a broader spectrum of malignant epithelia.

Our preliminary studies have indicated RPL19 to be differentially expressed in aggressive prostate cancers. Therefore, to test the hypothesis that increased expression of the RPL19 gene accurately identifies prostatic malignancy and can discriminate the progressive form of the disease, the expression status...
of this gene was analyzed in a wide range of prostatic cell lines and tissues by Northern blot hybridization analysis and by in situ hybridization (ISH). The relationship among RPL19 expression, degree of malignancy, and patient survival was assessed to determine its prognostic significance in human prostate cancer.

**Materials and Methods**

**Cell lines.** Human prostate cell lines used were the benign PNT2 cell line, the weakly malignant cancer cell line LNCaP, and the highly malignant cell lines DU145, PC3, and PC3M. Cells were grown as monolayer cultures in RPMI 1640 (Invitrogen, Paisley, United Kingdom) supplemented with 10% (v/v) FCS (Invitrogen), penicillin (1,000 units/mL), streptomycin (100 µg/mL), and t-glutamine (2 mmol/L). For the LNCaP cells, testosterone (5 ng/mL) and hydrocortisone (5 ng/mL) were added to the media.

**Human tissues.** Human prostatic tissues comprised an archival set held within the Pathology archive in the Department of Pathology, Royal Liverpool University Hospital, Liverpool, United Kingdom (11). This study was approved by the Liverpool Local Science Ethics Committee in accordance with the Medical Research Council guidelines. Normal and BPH tissue samples were obtained from prostates removed from men (average age = 52.4 years) undergoing primary cystoprostatectomy for invasive transitional cell carcinoma of the urinary bladder. Prostatic carcinoma tissues were obtained from men (average age = 55.4 years) from either needle core biopsies for primary diagnosis or trans-urethral resection of the prostate following radical retropubic prostatectomy. Mean preoperative PSA was 10.2 ng/mL. Mean follow-up time was 44 months. Tissue samples for Northern Blot analysis were excised from fresh radical retropubic prostatectomy specimens, weighed, and stored in RNA later buffer (Qiagen, Crawley, United Kingdom). All prostatic carcinoma tissue sections used for ISH were assessed according to internationally standardized conventions on Gleason grading (12, 13). Differentiation was graded as the sum of Gleason pattern scores. Specimens were divided into the three groups according to Gleason score: well differentiated (Gleason score = 2-5), moderately differentiated (Gleason score = 6-7), and undifferentiated (Gleason score = 8-10).

**RNA extraction.** Before extraction, tissue samples were lysed and disaggregated using a Kinematica Polytron homogenizer. Total RNA was extracted using Trizol reagent (Invitrogen) and analyzed using an Agilent BioAnalyzer to confirm the quantity and integrity of the extracted RNA according to profile and RNA integrity number.

**Northern hybridization.** Northern hybridizations were done to measure RPL19 mRNA levels in the five prostate cell lines and prostate tissues. Total RNA samples (15 µg each) were electrophoresed through a formaldehyde-agarose (0.8% w/v) gel, transferred onto a nylon membrane (Hybond N, Amersham, United Kingdom), and hybridized with a [32P]dCTP-labeled RPL19 cDNA probe. The radioactivity bound to the washed membranes was detected by exposure to Kodak X-AR-5 film for 24 hours. To standardize this probe, a radioactively labeled GAPDH probe was hybridized to the membrane after the RPL19. The relative intensities of the bands for a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe is used to standardize the hybridization.

**Microquantity differential display.** Differentially expressed genes in the benign and malignant cell lines were identified by microquantity differential display analysis of RPL19 expression profiles of different human prostate cell lines: benign PNT2 (lane 1) and malignant PC3M (lane 2). The arrow points to the band representing RPL19. Northern blot analysis of RPL19 expressed in PNT2 (lane 2), LNCaP (lane 2), DU145 (lane 3), PC3 (lane 4), PC3M (lane 5), benign prostate tissue (lane 6), and malignant prostate tissue (lane 7). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to standardize the hybridization. The quantification analysis of the levels of RPL19 mRNA in cell lines and tissues (lanes as B). The levels of RPL19 mRNA expressed in the benign cell line PNT2 and the benign tissue were set at 1. Columns, mean of three separate measurements; bars, SD.

**ISH.** RPL19 mRNA in human tissues was detected by ISH. A cohort of 126 formalin-fixed, paraffin-embedded tissue specimens was retrieved from the Pathology archive (Royal Liverpool University Hospital). All tissues had been fixed in 10% neutral buffered formalin for between 12 and 16 hours, processed through graded alcohol and xylene/chloroform before embedding in paraffin wax according to standard procedures. The 500-bp RPL19 cDNA fragment was obtained from reverse transcription-PCR of PNT2 total RNA using sense primer AGTATGCTACGGCTTCAGAAA and antisense primer TTCCTGTGTCATACCTGTC (14) and then cloned into pBluescript (Stratagene, Cambridge, United Kingdom). The RPL19 probe (antisense strand) was transcribed with T7 RNA polymerase (Roche, Penzberg, Germany), and the digoxigenin label was incorporated into the single-stranded probe using the Roche DIG RNA labeling kit. Tissue sections (4 µm) were cut and loaded onto glass slides coated with 3-aminopropyltriethoxysilane (Sigma, Poole, United Kingdom). Sections were taken in triplicate: for H&E staining, for ISH using the antisense RPL19 probe, and for control ISH using the sense RPL19 probe. ISH was done under RNase- and DNase-free conditions as previously described (15). Following hybridization, digoxigenin was detected using an alkaline phosphatase–conjugated sheep anti-digoxigenin antibody (Roche). ISH signals were generated using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Roche) to produce a blue-black precipitate at sites of hybridization.
Identification of RPL19 as a differentially expressed gene. Profile analysis of mRNA expression in the benign PNT2 cells and malignant PC3-M cells using microquantity differential display confirmed that one subset of the displayed cDNAs contained a band present in the cDNAs from PC3M cells but barely evident in the cDNAs from the PNT2 cells (Fig. 1A). Nucleotide sequence analysis of the corresponding cDNA fragment recovered from the denaturing gels revealed 100% homology to the cDNA coding for RPL19 (16) and confirmed this sequence to be a segment of RPL19.

Differential expression of RPL19 in prostate cell lines and tissues. Northern blot hybridization analysis (Fig. 1B) confirmed that relative to the level of RPL19 expression in the benign PNT2 cell line, expression levels in the malignant cells

<table>
<thead>
<tr>
<th>Tissues</th>
<th>No. cases</th>
<th>RPL19 staining intensities*</th>
<th>Total positive, N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8</td>
<td>8 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>BPH</td>
<td>32</td>
<td>15 (46.9)</td>
<td>9 (28.1)</td>
</tr>
<tr>
<td>GS &lt; 6</td>
<td>21</td>
<td>3 (14.3)</td>
<td>15 (71.4)</td>
</tr>
<tr>
<td>GS 6-7</td>
<td>25</td>
<td>3 (12.0)</td>
<td>5 (20.0)</td>
</tr>
<tr>
<td>GS 8-10</td>
<td>41</td>
<td>1 (2.4)</td>
<td>2 (4.9)</td>
</tr>
<tr>
<td>Carcinomas (total)</td>
<td>87</td>
<td>7 (8.1)</td>
<td>22 (25.3)</td>
</tr>
</tbody>
</table>

Abbreviation: GS, Gleason score.

Table 1. Detection of RPL19 mRNA in different types of prostate tissues by ISH

*RPL19 staining intensities were classified as negative (−), weakly (+), moderately (++), and strongly (+++) positive. The degree of malignancy of the carcinomas is shown by their combined Gleason scores.
were 6.7-fold higher in the LNCaP cell lines, 6.4-fold higher in DU145, 5.6-fold higher in PC3, and 4.9-fold higher in PC3M cells. Similarly, in matched samples of benign and malignant prostatic tissues from three patients, the mean level of RPL19 expression in the malignancies was 7.8-fold higher (range, 5.5- to 9.5-fold) when compared with the benign tissues. The results from patient one showing an 8.3-fold increase in malignant tissue compared with benign tissue is displayed in Fig. 1C.

**ISH Detection of RPL19 mRNA in different types of prostate tissues.** The sense control riboprobe for RPL19 was negative throughout the studies. The ISH data are detailed in Table 1. Comparison of staining intensities in the three types of tissue revealed that expression in carcinoma tissues was significantly greater than that observed in either BPH or normal tissues ($\chi^2: n = 127, P < 0.001$).

RPL19 staining intensities were further assessed in carcinomas that had been classified by Gleason score (Table 1). RPL19 staining intensities increased simultaneously with Gleason score. The intensities of staining observed in highly malignant tissues was significantly stronger than that in moderately malignant tissues (Goodman-Kruskal $\gamma: n = 66, P < 0.001$), which, in turn, stained with significantly stronger intensities than better differentiated tissues (Goodman-Kruskal $\gamma: n = 47, P < 0.0001$). Typical staining patterns of RPL19 mRNA ISH expression in 127 prostate tissue samples are illustrated in Fig. 2. In normal cells, staining occurred only in inflammatory cells. In BPH, RPL19 expression was localized to basal epithelial cells, whereas luminal epithelial cells were unstained. Low-grade (well differentiated) carcinomas exhibited mostly discrete nuclear staining of RPL19, whereas strong staining throughout the tissue was exhibited in both the cytoplasm and nucleus by highly malignant (poorly differentiated) carcinomas.

**RPL19 expression, Gleason score, and survival analysis.** Kaplan-Meier analysis assessed the relationship between RPL19 expression and patient survival. The 87 carcinomas were analyzed in the three groups of RPL19 staining intensities: negative and weak, moderate, and strong. Negative and weakly positive stained groups were combined due to the small sample number in the negative group. In addition, there was little difference in staining intensities between the negative (<10% cells staining) and weakly positive (10-30% cells staining) groups. The mean survival period was 51.9 months (range, 45.3-58.4 months) for the negative and weakly stained patient group, 49.0 months (range, 41.1-57.0 months) for the moderately staining patient group, and 37.3 months (range, 27.8-46.7 months) for the strongly stained patient group. Patients expressing higher levels of RPL19 had significantly poorer survival rates (log-rank test, $P < 0.05$). The relationship between cumulative probability of surviving and survival time after diagnosis is shown in Fig. 3B. At 60 months, 79.3% of patients with Gleason score 2 to 5 carcinomas survived, 73.1% of patients with Gleason score 6 to 7 (n = 25) groups, group 2, Gleason score 6 (n = 21); group 2, Gleason score 6 to 7 (n = 21); group 3, Gleason score 8 to 10 (n = 41) as defined in Materials and Methods.

**Discussion**

The data from this study confirm that the RPL19 gene is overexpressed in prostate cancer cell lines and primary prostatic carcinomas. The findings support the original hypothesis that expression of RPL19 is a powerful predictor of prostatic malignancy, and that the gene may be involved in prostate cancer progression. Northern blot hybridization showed RPL19 levels to be greatly increased in the malignant cell lines by 4.9- to 6.7-fold compared with the benign PNT2 cell line. In malignant prostatic tissue, the mean RPL19 level was increased by 7.9-fold compared with benign tissue.

Using ISH data from the archival set of prostatic tissue samples, Kaplan-Meier survival analysis revealed an inverse
relationship between elevated RPL19 expression levels and patient survival, in which patients expressing low levels of RPL19 survived significantly longer than patients expressing high levels \((P < 0.05)\). When Gleason score was used as the predictor for patient survival, an inverse relationship was also observed between the Gleason score and patient survival \((P < 0.05)\). Thus, RPL19 expression levels independently predict patient outcome as accurately as Gleason score.

RPL19 belongs to a family of some 70 ribosomal proteins organized into large and small ribosomal subunits. The proteins are synthesized in the nucleus and exported to the cytoplasm via nuclear pores, where they are believed to function as RNA chaperones during translation, coordinating interaction between the ribosome and RNA \((17, 18)\). The cell cycle is a highly regulated process such that abnormal expression of ribosomal proteins, or their inappropriate expression, may cause disturbances in protein translation. This may result in increased synthesis of oncogene products and decreased synthesis of tumor suppressor products, thus leading to initiation or promotion of malignancy.

Kaplan-Meier analysis of these data showed RPL19 expression to be as sensitive as Gleason score in predicting patient outcome. The Gleason score of a prostate tissue sample is assessed following a needle biopsy, which, as a surgical procedure, may cause the patient pain and discomfort. A more convenient and less invasive route to diagnose disease would be via blood or urine. As yet, it is unknown whether RPL19 can be detected in the blood. However, the ribosomal protein metallopanstimulin \((S27)\) has been identified in sera and used in the prognostic assessment of cancers including lung, head and neck, and colorectum \((19)\). Therefore, if present, RPL19 may be a valuable marker in the blood for prostate cancer.

Overexpression of other ribosomal proteins, including L7a and L37 mRNA, has been implicated in malignant prostate tissues \((20)\), although the biological significance of these elevated levels is unclear. No correlation has been provided between expression and biological behavior of individual prostate cancers, such as that reported herein.

The findings of this study support the original hypothesis that increased expression of RPL19 message not only identifies malignancy in prostatic epithelium but also independently predicts, with a high level of accuracy, the biological behavior of individual prostate cancers and patient survival. RPL19 may provide a valuable, novel marker of prostatic malignancy.

Acknowledgments

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References

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