Clinical Validation of Candidate Genes Associated with Prostate Cancer Progression in the CWR22 Model System using Tissue Microarrays

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Abstract

To explore molecular mechanisms of prostate cancer progression, we applied tissue microarrays (TMAs) to analyze expression of candidate gene targets discovered by cDNA microarray analysis of the CWR22 xenograft model system. A TMA with 544 clinical specimens from different stages of disease progression was probed by mRNA in situ hybridization and protein immunohistochemistry. There was an excellent correlation (r = 0.96; n = 16) between the expression levels of the genes in the xenografts by cDNA microarray and mRNA in situ hybridization on a TMA. One of the most highly overexpressed genes in hormone-refractory CWR22R xenografts was the S100P gene. This gene, coding for a calcium signaling molecule implicated in the loss of senescence, was also significantly associated with progression in clinical tumors by TMA analysis (P < 0.001), suggesting dysregulation of this pathway in hormone-refractory and metastatic prostate cancers. Conversely, two genes that were down-regulated during tumor progression in the CWR22 model system were validated in vivo: crystallin μ (CRYM) and a LIM-domain protein LMO4 both showed significantly lower mRNA levels in hormone-refractory tumors as compared with primary tumors (P < 0.001). These results illustrate a strategy for rapid clinical validation at the mRNA and protein level of gene targets found to be differentially expressed in cDNA microarray experiments of model systems of cancer.

Introduction

The growth of human prostate cancers is highly androgen dependent. However, despite an early favorable response to androgen withdrawal therapy, resistance to such hormonal therapies is almost inevitable, resulting in incurable androgen-independent tumors and metastases (1). The CWR22 human prostate carcinoma xenograft (2–5) model mimics clinical prostate cancer progression and provides an excellent model system for the discovery of genes associated with the relapse and therapy failure of human prostate cancer. cDNA microarray analyses of this model system have led to the discovery of several genes associated with disease progression (6–8). For example, in our recent analysis of 6605 genes by fluorescent cDNA microarrays, we found 112 genes that were up-regulated and 141 genes that were down-regulated in androgen-independent prostate cancer xenografts (6). Although xenograft model systems are invaluable for the gene discovery work as well as for experimental therapeutics, there is concern that growth of human cancer cells in an immunocompromised mouse host may not always be representative of the progression of cancer in patients (9). TMA technology facilitates rapid translation of cDNA microarray findings to clinical specimens using immunohistochemical analysis of protein expression in large numbers of clinical specimens (10). However, antibodies that would work on formalin-fixed tissues are often not available for genes discovered from cDNA microarrays. Here, we describe a strategy for gene target validation using both mRNA ISH and protein IHC. The assays were validated by inserting the same tissue specimens used in cDNA microarrays into the TMA format. We then applied this strategy to explore the expression of the top candidate genes and proteins discovered to be involved in the prostate cancer progression by cDNA microarray analysis of the CWR xenograft model system. Of all the genes on the cDNA microarray, the S100P gene showed the largest increase of mRNA levels in the recurrent tumors. Similarly, LMO4 and CRYM were among the most down-regulated genes following CWR22R progression, suggesting that they may also play a role in clinical tumor progression, making them interesting candidate gene to explore in clinical specimens.

Materials and Methods

Xenografts. Fresh frozen tissue from CWR22 human prostate cancer xenografts (2) was obtained from 13 different mice at different stages of hormonal therapy and tumor progression. For the studies here, four primary untreated CWR22 strains and four independent hormone-refractory CWR22R strains were analyzed. The LNCaP (American Type Culture Collection) cell line was used as a common reference and cultured in RPMI 1640 and 10% fetal bovine serum (Life Technologies, Inc., Rockville, MD) at 37°C and 5% CO2. mRNA was extracted with the FastTrack 2.0 Kit (Invitrogen Corp., Carlsbad, CA).

cDNA Microarray Analysis. Alterations in gene expression in a pool of recurrent tumors relative to a pool of primary tumors were identified by cDNA microarray analysis. Briefly, mRNA isolated from fresh frozen xenografts was used to make labeled cDNA, which was then hybridized on to glass slides containing arrayed cDNA fragments representing over 6000 individual genes. The experiments and the methods and analysis of the data are described in detail elsewhere (6).

TMA Construction. A prostate TMA was constructed from formalin-fixed paraffin-embedded tissue specimens as described previously (7, 10) and consisted of 544 specimens that represented controls and various stages of prostate cancer progression. These included 45 specimens of benign prostatic hyperplasia as controls, 60 specimens with prostatic intraepithelial neoplasia, 264 primary tumors, 41 distal metastases, and 134 tumors classified as hormone refractory also have metastases. Although tumors in the metastatic group have not been characterized for primary tumors, 41 distal metastases, and 134 tumors classified as hormone refractory. The two latter groups are inter-related. Many, but not all, of the tumors that have been classified as hormone refractory also have metastases. Although tumors in the metastatic group have not been characterized for response to hormonal therapy, some of them may display hormone independence. Therefore, hormone-refractory tumors and metastases are both considered to be the most advanced stage of the disease (without a distinction of...
which one is more progressed). Additionally, 28 xenograft tumors representing primary CWR22, regressing CWR22, and recurrent hormone-refractory CWR22R were included as controls (a total of 572 prostate specimens). For 16 of these xenografts, we had gene expression data from cDNA microarrays, making it possible to compare mRNA and protein levels measured for the target genes in the same specimens using cDNA and TMA techniques.

**Immunohistochemical Analysis of TMAs.** Antigen retrieval was performed by treatment in a pressure cooker for 30 min. Standard indirect immunoperoxidase procedures were used for IHC (Envision Plus; DAKO). A monoclonal mouse antibody (1:1000; Transduction Laboratories, Lexington, KY) was used for detection of S100P. The reactions were visualized with diaminobenzidine. Staining intensity was independently classified into three categories (negative, weak, and strong staining) by two pathologists (G. H. and W. F.).

**mRNA ISH Analysis of TMAs.** Eight oligodeoxynucleotide probes (40–45 bp each) were designed, spanning the length of the coding region of each of the candidate genes tested. Each probe was labeled separately using terminal deoxynucleotidyl transferase incorporating [α-32P]dATP as a 3′ tail, purified by Qiagen columns (Qiagen, Valencia, CA), and pooled for hybridization. After pretreatment of TMA sections and overnight hybridization, the sections were dried and exposed using a Fuji phosphorimager screen. Screens were scanned using a Fuji Bio-imaging analyzer 5000 using 25 μm resolution. The intensity of the signal was quantified using MacBAS v2.5 image software (Fuji Photo Film Co., Ltd.)

**Results**

Comparison of levels of expression for 6605 genes in primary and recurrent prostate cancer CWR22 xenografts is presented in Fig. 1 in the format of a scatter plot and a distribution of ratios. In general, most genes were expressed at the same level before and after progression to recurrence. Expression levels of 141 transcripts were significantly lower, and expression levels of 112 transcripts were higher in the recurrent tumors as compared with the primary tumors (Ref. 6; Fig. 1).

One of the most highly overexpressed genes in the recurrent prostate cancers was S100P (Figs. 1 and 2), coding for a calcium signaling protein previously associated with senescence in breast cancer cell lines. We decided to evaluate whether S100P is also expressed in clinical prostate cancer specimens, what the frequency of its expression is in large patient materials, and whether its expression is associated with prostate cancer progression *in vivo*. We applied the TMA technology for the rapid molecular pathological analysis of S100P at both the mRNA and protein levels in 544 clinical prostate cancer specimens at different stages of tumor progression. Xenograft specimens that were originally used in the cDNA microarray analyses were fixed in formalin, embedded in paraffin, and inserted into the same TMA block as controls for the mRNA ISH (Fig. 2A).

Results from cDNA microarray, Northern blotting, and mRNA ISH were all highly concordant in revealing expression levels of the S100P gene, with the highest levels seen in recurrent CWR22R xenografts (Fig. 2A). Furthermore, protein levels measured by IHC on CWR22 xenograft specimens on the TMA were also in line with the three mRNA-based assays. These experiments validated the mRNA ISH and protein IHC assays on the TMA format (Fig. 2A).

**Discussion**

Functional genomic analyses of cancer usually reveal hundreds of candidate genes that may provide clues to the molecular pathogenesis of disease or that represent candidate therapeutic and diagnostic gene targets. cDNA microarray analysis is typically performed from a few dozen cancer specimens or, in the case of model systems, from just individual cell line or tumor specimens. TMA technology is a powerful follow-up experiment to cDNA microarray analyses in the clinical translational studies of newly defined genes (7, 11–14). As shown in this study, TMA analysis facilitates (a) validation of the findings in *vivo* in clinical specimens, (b) ascertainment of the frequency of molecular alterations in large numbers of patient specimens from different stages of tumor progression, (c) determination of the clinicopathological associations for the candidate cancer targets, (d) ensuring translation of the mRNAs to proteins, and (e) study of the cellular and subcellular localization of the target genes and proteins. Hundreds of genes were associated with prostate cancer progression and therapy failure in the CWR22 experimental model system. A specific *in situ* assay was developed and validated here for the TMA analysis of S100P, a gene coding for a calcium signaling protein (15, 16) that is significantly up-regulated in hormone-refractory CWR22R pros-
Fig. 2. A, validation of in situ assays for S100P mRNA and protein expression on a TMA format. The cDNA microarray spots representing the S100P gene are shown from nine independent hybridizations. Quantitation of S100P mRNA by ISH and S100P protein by IHC of the same nine xenografts on a TMA is also shown. Northern blotting validated the cDNA microarray analysis, and concordance with in situ quantitation is illustrated by the line graph. B, IHC analysis of S100P protein expression was conducted on a prostate cancer progression TMA consisting of 544 clinical prostate cancer specimens, of which 440 were analyzable. An overview image of the IHC stain on one part of the TMA shows differential expression of the S100P protein. The bar graph on the right shows the frequency of samples that had strong S100P staining (score of 3 or 4) for each of the human prostate tumors at different stages of progression on the TMA. Although the figure depicts a linear progression of prostate cancer, benign prostatic hyperplasia should be considered more like a control group than a precursor lesion for cancer. Furthermore, hormone-refractory tumors and metastases are both considered to be advanced, lethal forms of cancer. These states are inter-related, and the order of progression to these steps may vary from one patient to another.
tate cancer specimens, and for two down-regulated transcripts, CRYM and LMO4.

Because antibodies are often not available for gene products discovered from cDNA microarray surveys, it is essential to detect these transcripts on TMAs using mRNA ISH (10, 17). Here we designed a strategy for validating mRNA ISH-based assay of transcript abundance by inserting into the TMAs the same specimens that were originally used in the cDNA microarray analyses. There was an excellent correlation between mRNA ISH and cDNA microarray results, validating that these particular transcripts could be measured accurately by mRNA ISH in formalin-fixed tissues in a TMA format. mRNA ISH was performed with several radioactively labeled oligonucleotide probes for different regions of the target genes. The use of short probes to different regions of the genes made it possible to obtain a signal even from degraded mRNAs that inevitably exist in clinical specimens. Finally, because an antibody was also available for S100P, we showed how the increased mRNA levels of S100P were translated to increased protein content as visualized by IHC on the clinical specimens. Increased S100P RNA and protein expression measured from the LMO4 element on 8 different cDNA microarrays for each of the tumors was plotted as a bar graph together with the intensity of mRNA ISH (18) for 16 TMA elements (the 8 xenograft tumors punched in duplicate). The inset line graph indicates that there is a high correlation (r = 0.96) between the methods of quantitating mRNA levels. The TMA bars 1–6 represent three different primary CWR22 xenografts, and bars 7–16 represent five different recurrent CWR22R xenografts. Except for a single primary xenograft (bars 5 and 6), the primary xenografts had higher levels of LMO4 mRNA than the recurrent tumors.

References


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Fig. 4. LMO4 and CRYM mRNA was measured by ISH on a TMA format. Signal intensity from radioactive signals on either a xenograft or a patient TMA was plotted for each prostate cancer involves systematic restoration of androgen responsive genes and activation of rapamycin sensitive signaling. Oncogene, 20: 6718–6723, 2001.


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