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

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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 \(\mu\) (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 microarray analyses. 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% CO\(_2\). 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. 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 diamino-benzidine. 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-33P]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 recurrent xenografts at the 99.9% confidence level. For 16 primary CWR22, regressing CWR22, and recurrent hormone-refractory CWR22R tumors relative to primary CWR22 in cDNA microarray analyses. In both cases, mRNA ISH on TMAs validated the relative expression levels seen by cDNA microarrays in the CWR22 xenograft specimens (Figs. 3 and 4). Direct comparison of the measurement of gene targets by cDNA microarray and mRNA ISH on a TMA format was also performed. For example, the levels of LMO4 mRNA measured by mRNA ISH on a TMA and by cDNA microarrays were highly correlated (Fig. 4). This analysis revealed a lower level of LMO4 and CRYM expression in 17 recurrent CWR22R xenografts (P < 0.001) as compared with 19 primary CWR22 xenografts (Fig. 4).

A significant decrease (P < 0.001) in mRNA levels was observed for both LMO4 and CRYM during tumor progression in cancer patients by mRNA ISH on the TMA (Fig. 4). Fig. 4 compares the expression levels of LMO4 and CRYM in 185 primary prostate cancers with the expression levels in 89 hormone-refractory prostate cancers. The mean intensity of actin mRNA was used as a negative control in the mRNA ISH (data not shown). Comparison of mRNA ISH levels between primary and hormone-refractory tumors on the same array revealed no significant differences between the two groups (P = 0.927; nonsignificant).

**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-

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**Fig. 1.** cDNA microarray data analysis of hormone-refractory prostate cancer xenografts. The histogram shows the distribution of relative ratios and that 253 genes are differentially expressed between primary and recurrent xenografts at the 99.9% confidence level. For the same data, the scatter plot illustrates the relative expression of three top candidates.
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 TMAs. Increased S100P RNA and protein expression measured from the clinical specimens on the TMAs also showed a significant association with tumor progression. Conversely, CRYM and LMO4 were down-regulated in clinical specimens from hormone-refractory tumors, which is in line with the cDNA microarray results in the CWR22 xenograft model system.

S100P belongs to the S100 family of proteins that are characterized by EF-hand Ca$^{2+}$ binding motifs (15, 16). This small 95-amino acid protein binds divalent ions including calcium and magnesium, which have an effect on both protein conformation and homodimerization (15, 16). It has been hypothesized that S100P may be involved in mediating calcium signaling, although the signaling pathways are not yet known. In addition, in a screen for genes induced during loss of senescence, S100P was found to be one of the genes elevated following loss of senescence in breast cancer cell lines (18). It has also been identified in screens for genes that are regulated by androgens in prostate cancer cells, although an association with hormone-refractory prostate cancer has not been reported previously (8, 19). Finally, S100P expression was elevated in doxorubicin-resistant cell lines (20), further implicating its role in therapy failure by possibly regulating cell survival. These previous observations, taken together with the experimental and clinical associations presented in this study, therefore suggest both a candidate gene target and an entire pathway that could be used therapeutically, which are consistent with the reported importance of calcium signaling (15, 16, 18) in prostate cancer cell survival. Further investigations of the therapeutic value of S100P and the biological significance of associated pathways in conferring androgen-independent cell growth and tumor progression in prostate cancer are warranted.

LMO4 is a member of the LIM-only (LMO) subfamily of LIM domain-containing transcription factors that are expressed during embryonic development (21, 22). CRYM codes for a thyroid hormone-binding protein (23, 24). The expression levels of both LMO4 and CRYM were decreased during clinical progression of prostate cancer in patients. A role in prostate cancer progression has not been reported previously for either of these genes. Although the putative mechanism of action of these genes in prostate cancer is unknown, we have observed that both LMO4 and CRYM mRNA levels are induced following anticancer drug suppression of prostate cancer cells in vitro, further implicating the expression of these genes in the growth and survival of prostate cancer cells. Our clinical observations warrant functional investigation of the role of the CRYM and LMO4 genes in prostate cancer and tumor progression.

In conclusion, we showed how the TMA technology enabled validation of the involvement of three putative gene targets in clinical prostate cancer progression. A role of these genes was hypothesized based on a functional genomic analysis of xenograft tissues. We also present a strategy for development and validation of in situ assay methods for such candidate gene targets identified in high-throughput functional genomic screens of model systems of cancer. The method is based on the inclusion of the specimens analyzed by cDNA microarrays in the TMAs. Large-scale development of mRNA ISH-based assays for novel gene targets would significantly facilitate translational studies of candidate target genes, provided that reliable assays for such targets can be generated and validated. Alterations in S100P, CRYM, and LMO4 genes are now proven to be involved in the acquisition of androgen-independent growth and failure of therapy in prostate cancer patients.

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References


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