ABSTRACT

NKX3.1 is a prostate-specific homeobox gene located on chromosome 8p21. In the mouse, Nkx3.1 has growth-suppressive and differentiating effects on prostatic epithelium. Mutations of the coding region of NKX3.1 were not found in human prostate cancer, failing to support the notion that NKX3.1 was a tumor suppressor gene. To study the expression of NKX3.1 protein in human tissues and prostate cancer, we derived a rabbit antiserum against purified recombinant NKX3.1. Among normal human tissues, NKX3.1 expression was seen in testis, in rare pulmonary mucous glands, and in isolated regions of transitional epithelium of the ureter. NKX3.1 was uniformly expressed in nuclei of normal prostate epithelial cells in 61 histological sections from radical prostatectomy specimens. We analyzed 507 samples of neoplastic prostate epithelium, most of which were contained on a tissue microarray that contained samples from different stages of prostatic neoplasia. We observed complete loss of NKX3.1 expression in 5% of benign prostatic hyperplasias, 20% of high-grade prostatic intraepithelial neoplasias, 6% of T1a/b samples, 22% of T3/4 samples, 34% of hormone-refractory prostate cancers, and 78% of prostate cancer (P < 0.0001).

INTRODUCTION

NKX3.1 is a homeobox gene with prostate-specific expression in the adult (1). NKX3.1 maps to chromosome 8p21, a region that undergoes LOH in ~75% of prostate cancer specimens (2–6). For this reason, NKX3.1 was a candidate target gene for disruption by the tumor suppressor gene. Loss of the expression of this growth suppressor gene has potent growth-suppressing and differentiating effects on prostatic epithelium. Mouse knockout mice have abnormal prostate morphology with overgrown and dysplastic epithelium (7). Disruption of prostate epithelial morphology and dysplasia is more severe in Nkx3.1-null mice (7). The suggestion that gene dosage, and therefore the amount of protein, may be important for the growth-suppressor effects of NKX3.1 prompted us to study its expression in human prostate cancer specimens.

This report describes the derivation of an antiserum against purified recombinant NKX3.1 protein and the immunohistochemical expression of NKX3.1 in normal human tissues and in prostate cancer specimens. One report of NKX3.1 mRNA expression in human prostate cancer tissues described increased expression in prostate cancers compared with adjacent normal tissue (8). Our data examining NKX3.1 protein expression support the opposite conclusion. We demonstrate that loss of the expression of this growth suppressor correlates with prostate tumor progression.

MATERIALS AND METHODS

Expression and Purification of NKX3.1 Recombinant Protein. A 3’-truncated cDNA of wild-type NKX3.1, including nucleotides 1–581 and excluding the region that coded for the COOH-terminal region of the protein downstream from the homeodomain, was inserted into pMAL-C2g vector (New England Biolabs, Waltham, MA) at the NdeI and EcoRI restriction sites. Fusion plasmid was transformed into BL-21-compliant cells (Stratagene, La Jolla, CA). An overnight culture of bacteria containing the fusion plasmid was induced with 0.5 mM IPTG for 2 h. MBP*-NKX fusion protein was purified by affinity chromatography with amylose resin (New England Biolabs). Purified fusion protein was cleaved with 0.05 μg of genenate I (New England Biolabs) per 10 μg of fusion protein at room temperature for 24 h. Pure recombinant NKX3.1 was purified again by DEAE ion exchange chromatography.

Production of NKX3.1 Polyclonal Antibody. Twenty-five μg of purified NKX3.1 recombinant protein in TiterMax adjuvant emulsion (CyRx Corpora- tion, Norcross, GA) were inoculated into New Zealand White rabbits. The total volume of the initial inoculation was 400 μl, and a 200-μl boost was administered 3–4 weeks later. Rabbits were test bled 3–4 weeks after the initial inoculation and after the second boost. Rabbit anti-NKX3.1 antibody was purified by affinity chromatography by successive passes through BL-21-MBP CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala Sweden) followed NKX3.1 CNBr-activated Sepharose 4B.

Immunohistochemical Staining. Deparaffinized tissue sections were preheated in 10 mM sodium citrate solution for 20 min in a Black and Decker vegetable steamer. NKX3.1 antibody diluted 1:1000 in blocking buffer (1:70 dilution of goat serum in PBS) was incubated on slides for 1 h at room temperature. Sections were then incubated with 1:200 diluted biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 30 min and ABC solution (Vector Labs) for another 30 min. VIP peroxidase substrate (Vector Labs) was used to stain tissues, which were then counterstained with methyl green.

Tissues. Sections of normal human tissues were obtained from the Lombardi Cancer Center Histopathology and Tissue Shared Resource. Prostate tissue specimens for normal tissue and the 30 prostate cancers in the validation set came from the Lombardi Cancer Center Histopathology and Tumor Core Laboratories. These specimens were collected at the time of RP and therefore represent specimens from clinical stage I and II prostate cancers. All histological diagnoses were confirmed.

Keywords: NKX3.1, prostate cancer, homeobox, LOH, immunohistochemistry, prostate cancer progression.
identified 477 tissue core specimens that were included in the analysis. Tissue specimens were available from 30 radical prostatectomies, 43) primary tumors with stage T1a/b according to International Union Against Cancer criteria (10), incidentally discovered after transurethral resection for presumed BPH (n = 35); primary, locally advanced tumors (clinical stage T3/4) treated by transurethral resection (n = 27); distant metastases collected from autopsies of patients who had died from end-stage metastatic prostate cancer (n = 35); and 108 local recurrences after hormonal therapy failure, including 65 transurethral resections from living patients and 43 specimens obtained from autopsies. Tumor grading on the original tissue sections was performed according to Gleason (11). The array also included 54 cores from high grade PIN lesions; however, because of the focal nature of PIN, we verified the H&E staining of each sample on the array and identified only 20 as clearly showing high-grade PIN in the tissue core specimens on the array.

**Statistical Methods.** Specimens were available from 30 radical prostatectomies. These specimens were assessed for Gleason score and NKX3.1 expression to determine whether NKX3.1 expression differed among specimens with at least one Gleason grade ≥4 compared with those with both grades <4. The prostate tissue samples available for tissue microarray analysis were ordered by increasing disease severity for the following classifications: BPH, PIN, T1 tumors, RP specimens, and T3/4 tumors. Specimens available from HR samples and metastatic disease represented more severe disease than the previously mentioned tissues, but their position in severity status relative to each other was unknown. Of primary interest was whether there is a decrease in NKX3.1 expression with increasing disease status. Two separate questions were addressed. The first was whether a trend in NKX3.1 expression is present with disease status BPH through T3/4 in the order listed above, with HR tumors as the most severe disease status. The second question was similar, except that metastatic disease rather than HR tumors was the most severe disease. Of additional interest was whether the combined group of T1a/b and RP tissues differed from T3/4 and whether it differed from metastatic tumors. These questions were tested using a Jonkheere-Terpstra test as implemented in StatXact (Cytel). Unless specified below, all tests were considered significant if P was <0.05. To control for the two tests using HR or metastatic tumors as the sixth tissue type, the decrease in NKX3.1 expression was considered significant if the two-sided P was <0.025. Specific pairwise comparisons with BPH through T3/4 were performed for HR or metastatic tumors provided the overall test was significant. Similarly, the two comparisons of T1a/b with either T3/4 or metastatic tissue were considered significant for P < 0.025.

**RESULTS**

Recombinant NKX3.1 was made as an MBP fusion protein in *Escherichia coli*. Cleavage of the fusion protein with genenase yielded electrophoretically pure NKX3.1 (Fig. 1A). Antibody to MBP did not react with cleaved NKX3.1, indicating complete cleavage of NKX3.1 from the fusion protein (Fig. 1B). Rabbit antiserum derived against purified recombinant NKX3.1 reacted only with NKX3.1 on Western blot and not with either *E. coli* proteins or MBP (Fig. 1C). The antiserum recognized 32-kDa NKX3.1 in TSU-Pr1 cells transfected with an NKX3.1 expression plasmid, but detected no proteins in TSU-Pr1 cells because they express <1/100 the level of NKX3.1 mRNA found in LNCaP cells (Fig. 1D). The induction of NKX3.1 mRNA by androgen treatment of LNCaP cells has been described and was reflected in the induction of NKX3.1 protein after R1881 treatment of LNCaP cells (Fig. 1D; Refs. 1, 12).

The expression of NKX3.1 mRNA is restricted in the adult mouse and humans. In the mouse, expression is seen only in the prostatic lobes and the bulbourethral gland (7). Expression in humans was seen predominantly in the prostate, but low levels of mRNA were also detected in testis (1). There also appeared to be signals in peripheral blood lymphocytes (1). In fact, our attempts to use NKX3.1 as a prostate-specific marker failed because reverse transcription-PCR detected transcripts in female peripheral blood blood.

To clarify the expression of NKX3.1 in human tissues, we did immunohistochemical staining of 16 human tissues, including prostate, brain, heart, lung, kidney, testis, bladder, ureter, skin, liver, spleen, bone marrow, small and large intestine, breast, and endometrium, using our rabbit antiserum. On the basis of preliminary studies with LNCaP cells and NKX3.1-transfected TSU-Pr1 cells, we expected to find this homeobox protein localized to the nucleus.

We detected expression of NKX3.1 in testis, confirming the results for mRNA expression (Fig. 2, A and B). We also noted expression of NKX3.1 in rare mucous glands of the lung (Fig. 2, C and D). Lastly, we found expression of NKX3.1 in groups of ureteral epithelial cells periodically spaced along the lumen of the ureter (Fig. 2, E and F). Bladder transitional epithelium contained rare single cells with nu-

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6 H. J. Voeller and E. P. Gelmann, unpublished data.

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clear staining (not shown). We found no expression in tissues that contained blood cells, including bone marrow and spleen. Nonmalignant prostatic epithelial cells had uniformly positive nuclear staining for NKX3.1. This was seen in 61 RP specimens (Fig. 2, G and H).

To determine the expression of NKX3.1 in neoplastic prostate epithelium, we analyzed a tissue microarray that contained a spectrum of tissue samples providing a cross-section of prostate tumor progression. To validate the data in the array from one category of tissue, we performed conventional immunohistochemical staining on a separate set of 61 embedded tissue blocks from prostatectomy specimens. Thirty of these blocks contained malignant foci that were scored for NKX3.1 expression and compared with the results of the RP samples in the tissue microarray.

Neoplastic prostate epithelium was found to display three different patterns of immunostaining for NKX3.1 expression. Many samples stained uniformly for NKX3.1. Some samples stained heterogeneously, with some malignant cells stained and adjacent cells not stained. Some samples displayed no staining for NKX3.1. In samples in which malignant cells did not express NKX3.1, adjacent normal epithelial cells were invariably positive, providing an internal control for the quality of the specimen. The patterns of staining are shown in Fig. 3. For the purposes of analyzing the 477 microarray samples and the 30 sections, uniform staining was awarded a score of 2, heterogeneous staining a score of 1, and samples that did not stain were scored 0.

The results of staining for the tissue microarray are shown in Table 1. Whereas the majority of samples from early-stage cancers stained uniformly for NKX3.1 in epithelial cell nuclei, the number of samples with heterogeneous or negative staining increased among the locally advanced T3/4 and HR samples. There was a significant reduction of NKX3.1 expression in advanced prostate cancer as defined by either HR (P < 0.0001) or metastatic disease (P < 0.0001). HR demonstrated significantly lower NKX3.1 expression than BPH (P < 0.0001), T1 a/b (P < 0.0001), and RP (P = 0.013). Metastatic samples differed from all tissue samples BPH through T3/4 (P < 0.0001 for each). The combined group of T1 a/b tumors and RP samples had significantly higher NKX3.1 expression than metastases (P < 0.0001) but did not achieve a significant difference compared with T3/4 (P = 0.061; Refs. 13, 14).

Seventy-eight percent of metastatic samples had no staining for NKX3.1. The integrity of these autopsy samples was confirmed by staining for Ki-67, which was seen to decorate nearly all of the metastatic samples. Because Ki-67 may be more stable than NKX3.1 under conditions found at autopsy, we also compared the HR speci-

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**Fig. 2.** Immunohistochemical staining of normal human tissues with anti-NKX3.1. Sections were cut in parallel and stained with either H&E (A, C, E, and G) or anti-NKX3.1 (B, D, F, and H). Images A–F were captured digitally using ×400 microscopic magnification; G and H, were captured digitally using ×63 magnification. A and B, normal testis seminiferous tubules. C and D, pulmonary mucous gland. E and F, ureteral transitional epithelium. G and H, prostate gland. IHC, immunohistochemistry.
However, staining of HR autopsy specimens scored positive twice as often as staining of autopsy-derived metastases.

The series of 30 RP specimens analyzed as conventional sections cut from paraffin blocks gave nearly identical results to the distribution of staining patterns found among the RP specimens in the tissue microarray. The microarray RP samples gave a nearly identical distribution of staining scores compared to these 30 paraffin block samples; therefore, the staining of conventional samples validated the tissue microarray data and confirmed that a fraction of early-stage prostate cancers lose expression of NKX3.1.

The 30 samples from paraffin blocks were subjected to histological grading (11). In contrast to the relationship between loss of NKX3.1 staining and prostate tumor progression, we found no relationship between NKX3.1 staining scores and Gleason scores in the 30 RP blocks. The distribution of NKX3.1 staining results over the range of tumor grades as measured by Gleason score is shown in Table 2. Samples were compared across all scores and compared as groups with at least one grade $\geq 4$ versus both grades $<4$. There was no evidence indicating that patients with lower NKX3.1 expression had higher Gleason scores ($P = 0.611$; Ref. 13).

### DISCUSSION

Our data show that NKX3.1 expression is lost in a significant fraction of early-stage prostate cancer and that loss of expression correlates with tumor progression. Because NKX3.1 has differentiating and growth-suppressing effects in the mouse prostate, it is tempting to speculate that NKX3.1 plays a tumor-suppressor function in human prostate cancer. If, similar to the mouse, the human prostatic epithelium is sensitive to the level of NKX3.1 protein, then diminution in the level of NKX3.1 expression, even in tissues that demonstrate heterogeneous staining, may play a role in the pathogenesis or progression of human prostate cancer. NKX3.1 staining was found in three patterns in the tissue samples. Most samples, except for metastases, showed diffuse staining of both normal and neoplastic prostatic epithelial cells. In our experience, all normal prostate epithelial cells express NKX3.1. NKX3.1 expression decreases with disease severity. That fact combined with the lack of relationship between NKX3.1 expression and the Gleason score means that NKX3.1 expression has the potential to be a promising new prognostic marker if it is associated with patient survival. Further studies are warranted in this area.

A previous report that NKX3.1 mRNA expression was increased in prostate cancer tissues compared with adjacent normal tissues arrived at a different conclusion from our findings (8). If the results of Xu et al. (8) are confirmed, it would lead to the conclusion that control of NKX3.1 expression occurs at the posttranscriptional level.

In the survey of 61 tissue sections, we found no example of men that were obtained surgically with those obtained at autopsy. Among 128 HR specimens, 43 were autopsy specimens and 85 were surgical specimens. Of the HR autopsy specimens, 23 of 53 (43%) had staining scores of 0 compared with 24 of 85 (25%) surgical HR specimens. Therefore, it is probable that diminished staining of autopsy-derived material resulted, in part, from antigen degradation.

#### Table 1 Frequencies of progression array staining for NKX3.1

<table>
<thead>
<tr>
<th>NKX3.1 staining score</th>
<th>BPH</th>
<th>PIN</th>
<th>T1a/b</th>
<th>RP</th>
<th>T3/4</th>
<th>HR</th>
<th>Meta</th>
<th>$P_{\text{vs.}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>36 (84)</td>
<td>9 (45)</td>
<td>83 (76)</td>
<td>68 (62)</td>
<td>14 (52)</td>
<td>64 (50)</td>
<td>4 (10)</td>
<td>19 (63)</td>
</tr>
<tr>
<td>1</td>
<td>5 (12)</td>
<td>7 (35)</td>
<td>19 (17)</td>
<td>24 (22)</td>
<td>7 (26)</td>
<td>20 (16)</td>
<td>5 (13)</td>
<td>7 (23)</td>
</tr>
<tr>
<td>0</td>
<td>2 (5)</td>
<td>4 (20)</td>
<td>7 (6)</td>
<td>18 (16)</td>
<td>6 (22)</td>
<td>44 (34)</td>
<td>31 (78)</td>
<td>4 (13)</td>
</tr>
<tr>
<td>Total (100%) tissue</td>
<td>43 (85)</td>
<td>20 (40)</td>
<td>109</td>
<td>110</td>
<td>27</td>
<td>128</td>
<td>40</td>
<td>30</td>
</tr>
</tbody>
</table>

* RP samples from tissue microarray and paraffin sections presented similar NKX3.1 score profiles.
* Meta, metastatic disease.
* This test is significant at 0.05. No adjustment was made because these analyses were controlled by the overall test.
* This test is significant at the specified $P < 0.025$.  

Fig. 3. Immunohistochemical staining of prostatic tissue and tissue microarrays with anti-NKX3.1. A, examples of prostate cancer specimens from tissue microarrays stained with H&E (left) and corresponding section stained with anti-NKX3.1 (right). Staining patterns observed were diffuse staining (2), heterogeneous staining (1), or no staining (0). All tissues were scored according to this scheme, and the data are summarized in Table 1. B, parallel sections from a Gleason score 3 + 3 prostate cancer specimen stained with either H&E (left) or anti-NKX3.1 (right). Images were captured at $\times 100$. NKX3.1 expression was absent in the adenocarcinoma (upper left of slide) and present in hyperplastic glands (lower right of slide). IHC, immunohistochemistry.
The finding that NKKX3.1 expression was lost most often in metastases is consistent with the notion that metastatic disease is the most dedifferentiated state of prostatic cancer. It may also be that NKKX3.1 expression is under the control of prostate stromal cells. In murine tissue recombinants of neonatal epithelium and mesenchyme from the embryo and may play a noncritical role in the development of other organs. In the murine development, the only apparent action of Nkx3.1 pathology attributed to loss of expression of the SV40 T antigen (17–19). Whether the Nkx3.1 promoter may have application in prostate-specific gene therapy. The expression of NKKX3.1 in other tissues shown in this report will help to identify potential organs for side effects of treatments targeted to the prostate by the NKKX3.1 promoter.

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Loss of NKX3.1 Expression in Human Prostate Cancers Correlates with Tumor Progression

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