Down-Regulation of Homeobox Gene GBX2 Expression Inhibits Human Prostate Cancer Clonogenic Ability and Tumorigenicity

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Abstract

Previously, we have demonstrated that GBX genes, a homeobox-containing human family of DNA-binding transcription factors consisting of GBX1 and GBX2, are overexpressed in a panel of human prostatic cancer cell lines (i.e., TSU-prl, PC3, DU145, and LNCaP) compared to normal prostate. In the present studies, specific primer sets were designed for reverse transcription-PCR detection of the expression of GBX1 versus GBX2 in human prostate cancer. These studies demonstrated that the GBX2 gene, but not the GBX1 gene, is consistently overexpressed in this panel of human prostate cancer cell lines compared to normal human prostate. Using a quantitative-competitive PCR analysis, GBX2 mRNA was expressed as 3 \times 10^5 copies/µg RNA in normal prostate tissue and 4 \times 10^6 copies/µg RNA in the immortalized normal neonatal prostate epithelial cell line 267B-1, as compared to 6 \times 10^5, 5 \times 10^5, 3 \times 10^5, and 1 \times 10^5 copies/µg RNA in TSU-prl, DU145, LNCaP, and PC3 prostate cancer cell lines, respectively. To examine the importance of GBX2 expression for prostate cancer malignancy, GBX2-overexpressing TSU-prl and PC3 human prostate cancer cells were transfected with a eukaryotic expression vector containing an antisense GBX2 homeobox domain cDNA. Stable transfecant clones with 5-10-fold decreased levels of GBX2 mRNA expression were obtained. When tested in vitro, the clonogenic ability of the GBX2 antisense transfectants was reduced by approximately 50% in both cell lines. When implanted s.c. into nude mice, the tumorigenicity of the antisense GBX2 transfectants from both human prostate cancer cell lines was inhibited by more than 70% compared to the parental cells. These results suggest that expression of GBX2 gene is required for malignant growth of human prostate cells.

Introduction

There is an urgent need for the development of prognostic indicators to predict which prostate cancer patient with apparently localized disease actually has clinically undetectable micrometastatic cancer requiring additional systemic treatment (1). The down-regulation of expression of a series of metastatic suppressor genes encoding cell surface proteins (e.g., KAI1, CD44, and E-cadherin) is a candidate for such a prognostic indicator (1). This has raised the issue of the mechanism for such coordinated down-regulation during the acquisition of metastatic ability by prostate cancer cells. One class of possible master regulators for such repression is the homeobox family of transcription factors. Homeobox genes comprise a large family encoding positive and negative transcription factors of the helix-turn-helix motif, the expression of which regulates body plan and pattern formation during development and various aspects of cell regulation and differentiation (2, 3). Characteristic of these genes is the presence of a homeobox domain region. The homeobox domain is a conserved structural motif of nucleotides encoding a 60-amino acid residue polypeptide sequence that recognizes and binds to specific DNA sequence motifs (2). In humans, as well as in mice, there are 39 class I homeobox genes that are organized in four clusters designated A, B, C, and D. In humans, these clusters are located on chromosomes 7, 17, 12, and 2, respectively (4, 5).

Many of these homeobox genes have been found to be involved in malignant processes as a series of cell lineages. These include Hox-7.1 (6), Hoxa-7, Hoxa-1, Hoxb-7, and Hoxc-8, as well as homeobox genes Eev-1 and Cdx-1 (7). Expression of Hox-7.1 in myoblasts inhibits terminal differentiation and induces cell transformation (6). Recently, mutations in HOXD13 have been found in families affected by synpolydactyly, an inherited human abnormality of the hands and feet (8, 9). Overexpression of homeobox genes (i.e., Hoxa-7, Hoxb-7, Hoxa-1, Hoxc-8, Eev-1, and Cdx-1) induces NIH 3T3 cell transformation and tumorigenicity (7). Altered expression of the homeobox genes is observed also in various human carcinomas, including breast (10), colon (11), rectal gastric (12), and lung (13). Recently, we demonstrated that members of the GBX class of homeobox genes are overexpressed in TSU-prl, PC3, LNCaP, and DU145 metastatic prostate cell lines compared to the normal prostate (13). The GBX class of homeobox domain genes comprises six known members. These include GBX1 (14) and GBX2 (15, 16) in humans, Gbx-1 (17) and Gbx-2 (18) in mice, chicken Hox7 (19), and Xenopus laevis homeobox gene xgbx-2 (GenBank accession no., U04867). This GBX family of homeobox genes shares more than 97% sequence identity in the 60 amino acids of the homeobox domain (14, 15, 17, 18). In humans, the GBX1 and GBX2 genes are located on chromosomes 7q36 and 2q37, respectively (14). Using RNA in situ hybridization analysis of mouse embryos, expression of mouse Gbx2 RNA, which is a homologue to human GBX2, occurs during gastrulation and neurulation (20). Gbx2 RNA is expressed in the spinal cord, hindbrain, optic vesicle, and mandibular arch (20), suggesting that Gbx2 expression is associated with the development of the nervous system. In the past recent study, GBX2 was identified as a transcription factor, the expression of which in myeloblasts is regulated by the v-Myb oncoprotein encoded by the avian myeloblastosis virus (21). This recent study demonstrated that virally induced expression of GBX2 in myeloblasts resulted in enhanced autocrine growth stimulation (21).

In this report, we determined the levels of expression of GBX1 versus GBX2 in normal human prostate versus human prostate cancer cell lines. In addition, GBX2 expression in TSU-prl and PC3 human prostate cancer cell lines was decreased by expression of GBX2 homeobox domain cDNA in the antisense orientation to test what effect this down-regulation has on the clonogenic ability and tumorigenicity of these prostate cancer cells.

Materials and Methods

Cells. LNCaP, DU145, TSU-prl, and PC3 human prostate cancer cell lines were maintained in RPMI 1640 with 10% fetal bovine serum as described
Previously, 13, 267B-1 (22), an immortalized normal human neonatal prostatic epithelial line isolated as a single colony of pRSV-T-transfected NP-2a cells, was maintained in P4-BF medium (Biological Research Facility and Facility, Inc., Ijamsville, MD).

Construction of Antisense GBX Homeobox Domain. The cDNA of the GBX homeobox domain was generated by RT-PCR of RNA from TSU-prl cells using degenerate oligonucleotide primers to the homeobox domain as described previously (13). The GBX homeobox domain was ligated into pCDNA3.1- vector (Invitrogen, San Diego, CA), and the plasmid containing the GBX homeobox domain in the antisense orientation downstream from the cytomegalovirus promoter was selected for further study. For transfection, TSU-prl and PC3 cells were transfected with this antisense GBX or vector control plasmid by a lipofectamine (Life Technologies, Inc., Gaithersburg, MD) protocol according to the manufacturer’s instructions. Single transfected colonies were isolated and maintained in the presence of G418 as described previously (23).

RNA Preparation and Northern Blot Analysis. Total cellular RNA was isolated and Northern blot analysis was performed as described previously (13). Normal human prostate RNA was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Twenty μg of total RNA were used for the Northern blot. Hybridization was performed with GBX homeobox domain cDNA probe (13) labeled with [α-32P]dCTP by random primer method according to the manufacturer’s instructions (Amersham Corp., Arlington Heights, IL).

RT-PCR Assay. Five μg of total RNA were added to 0.5 μg of oligodeoxynucleotide primer and brought to a final volume of 20 μl. The primer-RNA mixture was combined with 200 units of Moloney murine leukemia virus reverse transcriptase, and the reaction was performed according to the manufacturer’s instructions (Life Technologies). Samples were stored at -20°C. Oligonucleotide primers specific for GBX1 (forward, 5'-AAGCTAAGGGAGCTGGGAC-3'; reverse, 5'-CTTGCTGTGCTGCTGCTTGC-3') and GBX2 (forward, 5'-CACACCATCTACGGAAGAAC-3'; reverse, 5'-ACTGCTGCTGACTCCTCGTA-3') were designed according to the published sequences (14, 15) so that each would amplify the GBX1 and GBX2 cDNA with a denaturing temperature of 94°C for 45 s, an annealing temperature of 68°C for 1 min, and a product of 72°C for 2 min for 35 cycles. As a control for RNA integrity, a separate portion of cDNA was amplified using primers specific for human GAPDH (forward, 5'-CTGTTGCAACGAGGCTTCC-3'; reverse, 5'-TACACCACTTCTGCAGTCA-3') according to the GAPDH sequences (24). The predicted PCR product for GAPDH was 214 bp. PCR products were separated by electrophoresis on a 1.5% agarose gel, and DNA fragments were visualized and photographed under UV light by ethidium bromide staining.

Cloning and Sequencing of the GBX1 and GBX2 PCR Products. Both of the PCR products (i.e., GBX1 and GBX2) were excised from the agarose gel and directly cloned into the TA cloning vector (Invitrogen). The cloned PCR products were confirmed by sequencing using the double-stranded DNA cycle sequencing system (Life Technologies) according to the manufacturer’s instructions.

Quantitative PCR Analysis. GBX2 RNA expression analysis was performed using a quantitative competitive PCR method (25) with a synthetic competitor that differed from the GBX2 cDNA by a small internal deletion. The GBX2 competitor containing a 79-bp deletion was generated by PCR amplification of TA vector containing the GBX2 PCR product, produced as described in the preceding section. Competitive PCR was performed in a total volume of 20 μl using a constant amount of cDNA that was coamplified with serial dilutions containing known numbers of copies of the competitor. The cDNA was produced via RT as described above for the RT-PCR assay. PCR conditions were the same as described above. The two products were amplified with the same efficiency because of the high similarity. The PCR products were separated by electrophoresis in a 3% agarose gel, and the bands were visualized by ethidium bromide staining and quantified by densitometric scanning. The absolute amount of the target cDNA expressed as copies/μg of starting RNA was quantified on the basis of the amount of competitor needed to produce an equivalent PCR signal. Differences in the amount of starting RNA and in reverse transcriptase efficiency in RT reactions were minimized by normalizing data in comparison to the expression of the housekeeping gene GAPDH.

Clonogenic Assay. The anchorage-dependent clonogenic ability, expressed as the percentage of cells capable of producing growing colonies on tissue culture plastic plates, was performed as described previously (26).

Tumorigenicity Assay. Five-week-old male nude mice (Charles River Breeding Laboratories, Boston, MA) received a s.c. injection of 1 x 106 TSU-prl cells or PC3 antisense transfectants or a s.c. injection of vector alone (controls). Mice were weighed and sacrificed 10 days after the TSU-prl injection and 60 days after the PC3 injection. The tumors were weighed.

Statistical Analysis. Values are expressed as means ± SE. Statistical significance was calculated by one-way ANOVA, followed by the Newman-Keuls test for multiple comparison, with P < 0.05 being considered significant.

Results and Discussion

GBX2 but not GBX1 is Overexpressed in Prostatic Cancer. We previously demonstrated that GBX genes are consistently overexpressed in a series of human prostatic cancer cell lines (i.e., TSU-prl, PC3, DU145, and LNCaP) compared to normal human prostate using a cDNA probe recognizing the GBX homeobox domain region (13). Because GBX1 shares high homology (81%) with GBX2 cDNAs in the homeobox domain region, this probe does not differentiate between the expression of GBX1 and GBX2. To semiquantitatively compare the level of GBX1 versus GBX2 expression, RT-PCR analysis was performed using two distinct oligonucleotide primers designed specifically to amplify either GBX1 or GBX2, but not both. As shown in Fig. 1, all of the human prostate cancer cell lines (i.e., PC3, LNCaP, DU145, and TSU-prl) and normal human prostate express GBX1; however, the expression of GBX1 is not consistently higher in the prostate cancers compared to normal human prostate. These results suggest that, whereas normal prostate and prostate cancer consistently express GBX1, GBX2 overexpression is not required for prostate malignancy. In contrast, the GBX2 gene is overexpressed in all of the human prostate cancer cell lines compared to normal prostate (Fig. 1), suggesting that overexpression of the GBX2 gene is consistently associated with prostate cancer. To determine this more precisely, the absolute amounts of GBX2 expression in these malignant cell lines and in normal prostate were determined via competitive PCR. In competitive RT-PCR, a constant amount of reverse-transcribed RNA is mixed and coamplified with variable known amounts of synthetic competitor that contains the same primer template sequences as the target but differs from the target cDNA for the
PC3 cells detected by competitive PCR. The amount of GBX2 expression in the parental and vector control transfecteds is 1 x 10^5 copies/μg of RNA compared to 1 - 2 x 10^4 copies/μg of RNA in the PC3 antisense clones, whereas the number of GBX2 molecules in the TSU parental and vector control is 6 x 10^3 copies/μg RNA compared to 8 - 10 x 10^3 copies/μg RNA in the TSU antisense clones.

**Effect of Antisense GBX2 Homebox Domain on Clonogenic Ability and *in Vivo* Tumorigenicity.** Tumorigenic parental, vector control, and antisense transfectants were cultured under identical conditions, and their clonogenic ability was determined *in vitro*. All of the antisense transfectants from both cell lines had a significantly decreased (approximately 50%) clonogenic ability (*i.e.*, decreased percentage of initially inoculated cells capable of producing continuously growing clones) compared to the vector controls (Table 1). The GBX2 mRNA expression in the antisense clones in both cell lines was only reduced by 5-10-fold compared to their parental cells, but GBX2 mRNA expression was not eliminated (Table 1). This may explain why a more complete inhibition of their clonogenic ability was not observed. To test the effect of down-regulation of the GBX2 expression on the tumorigenicity of the TSU-prl and PC3 human prostate cancer cells, stable transfectants expressing the antisense plasmids were inoculated s.c. into nude mice. As controls, parental and vector-alone transfectants were also injected. As shown in Table 2, all of the animals that received injection of either TSU-prl or PC3 antisense GBX2 transfectants showed a more than 70% suppression of their tumor weight compared to those that received injection of the parental cells. In contrast, no significant tumor suppression for either cancer was observed in the controls treated with Neo vector alone.

In summary, we have demonstrated that overexpression of GBX2 but not *GBXI* gene consistently occurs in human prostate cancer cell lines. In addition, using an antisense approach, the present studies provide direct evidence that GBX2 gene overexpression is an important requirement for the clonogenic ability and tumorigenicity of prostate cancer cells.

**Table 1** *In vitro* clonogenic ability of PC3 and TSU-prl parental, vector, and their GBX antisense transfectants

<table>
<thead>
<tr>
<th>Cell clones</th>
<th>Clonogenic ability (%)</th>
<th>Percentage inhibition of clonogenic ability</th>
<th>No. of copies of GBX2/μg RNA</th>
</tr>
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<tbody>
<tr>
<td>PC3 parental</td>
<td>16 ± 2</td>
<td>12</td>
<td>1 x 10^5</td>
</tr>
<tr>
<td>PC3 vector</td>
<td>14 ± 3</td>
<td>66</td>
<td>2 x 10^4</td>
</tr>
<tr>
<td>PC3-AS1</td>
<td>7 ± 2</td>
<td>63</td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>PC3-AS2</td>
<td>6 ± 3</td>
<td>69</td>
<td>1.4 x 10^6</td>
</tr>
<tr>
<td>PC3-AS3</td>
<td>5 ± 2</td>
<td>53</td>
<td>6 x 10^5</td>
</tr>
<tr>
<td>TSU-prl parental</td>
<td>21 ± 4</td>
<td>5</td>
<td>6 x 10^5</td>
</tr>
<tr>
<td>TSU vector</td>
<td>20 ± 5</td>
<td>48</td>
<td>1 x 10^5</td>
</tr>
<tr>
<td>TSU-AS1</td>
<td>11 ± 3</td>
<td>43</td>
<td>1 x 10^4</td>
</tr>
<tr>
<td>TSU-AS2</td>
<td>12 ± 4</td>
<td>30</td>
<td>8 x 10^4</td>
</tr>
<tr>
<td>TSU-AS3</td>
<td>13 ± 4</td>
<td>28</td>
<td>8 x 10^4</td>
</tr>
</tbody>
</table>

* Inhibition versus parental cells.

**Table 2** *In vivo* characteristics of TSU-prl and PC3 parental, vector, and GBX antisense transfectants

<table>
<thead>
<tr>
<th>Cell clone</th>
<th>Tumor weight (mg) at excision</th>
<th>Percentage inhibition of tumor weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3 parental</td>
<td>500 ± 200 (4)</td>
<td>20</td>
</tr>
<tr>
<td>PC3 vector</td>
<td>400 ± 150 (4)</td>
<td>70</td>
</tr>
<tr>
<td>PC3-AS1</td>
<td>150 ± 100 (4)</td>
<td>70</td>
</tr>
<tr>
<td>PC3-AS2</td>
<td>0 (4)</td>
<td>70</td>
</tr>
<tr>
<td>PC3-AS3</td>
<td>100 ± 100 (4)</td>
<td>80</td>
</tr>
<tr>
<td>TSU-prl parental</td>
<td>700 ± 200 (8)</td>
<td>7</td>
</tr>
<tr>
<td>TSU vector</td>
<td>650 ± 200 (6)</td>
<td>7</td>
</tr>
<tr>
<td>TSU-AS1</td>
<td>150 ± 100 (6)</td>
<td>79</td>
</tr>
<tr>
<td>TSU-AS2</td>
<td>180 ± 50 (6)</td>
<td>74</td>
</tr>
<tr>
<td>TSU-AS3</td>
<td>200 ± 100 (6)</td>
<td>71</td>
</tr>
</tbody>
</table>

* Inhibition versus parental cells.

**Numbers in parentheses, number of injection sites/group.

* P < 0.05.
GBX2 EXPRESSION IN PROSTATE CANCER

human prostate cancer cell lines. Presently, while the antibody that targets GBX2 specifically is being developed and will be used to evaluate whether GBX2 protein expression is consistent with GBX2 mRNA expression, the ability of GBX2 transgenic overexpression to down-regulate KAI1, CD44, and E-cadherin metastasis suppressor gene expression in the 267B-1 normal prostate cell line is being tested, as well as the effect of GBX2 overexpression on the tumorigenicity and metastatic ability of these cells. In addition, competitive PCR analysis is being performed in both primary and metastatic cancer specimens from prostate cancer patients to determine whether the level of GBX2 overexpression is an independent prognostic indicator of prostate cancer aggressiveness and thus overall patient survival.

References

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