Down-Regulation of the KAI1 Metastasis Suppressor Gene during the Progression of Human Prostatic Cancer Infrequently Involves Gene Mutation or Allelic Loss


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Abstract

The KAI1 gene, located on human chromosome 11p11.2, suppresses tumor metastasis when expressed in certain cancer cells. To evaluate whether dysregulation of KAI1 occurs during the progression of human prostatic cancer, protein expression, mutation, and allelic loss of KAI1 were analyzed using a tissue bank of 98 primary cancers and 32 metastases. By immunohistochemical staining, high levels of KAI1 protein are detected in the epithelial but not stromal compartment of normal prostatic tissue. In epithelial cells, KAI1 protein is expressed on the plasma membrane. KAI1 protein expression is down-regulated in more than 70% of the 49 primary prostatic cancers from untreated patients. In 10 such untreated patients, down-regulation of KAI1 protein occurred in all of the lymph node metastases examined. In 15 patients with metastatic disease who had failed androgen ablation therapy, more than 90% of the primary prostatic cancers had down-regulation, with 60% having no KAI1 protein expression. Primers derived from the sequences flanking each exon of KAI1 were used to analyze KAI1 mutation and allelic loss by the method of PCR-single-strand conformational polymorphism. Using this method, no point mutation or allelic loss was detected in metastases from 10 patients. No allelic loss was detected in an additional 34 primary and 12 lymph node metastases via microsatellite analysis using the marker D11S1344, which is located in the region of KAI1. These results demonstrate that KAI1 protein expression is consistently down-regulated during the progression of human prostatic cancer and that this down-regulation does not commonly involve either mutation or allelic loss of the KAI1 gene.

Introduction

Prostatic cancers vary widely in their clinical aggressiveness. Some cancers metastasize rapidly and kill the patient within a year of initial diagnosis, whereas others remain localized, never metastasizing during the lifetime of the patient (1). If truly localized, prostatic cancer can be cured by radical prostatectomy (2). However, if the cancer only appears to be localized but in reality has metastasized, then systemic therapy is required. Unfortunately, there is no prognostic method to identify patients with localized metastatic prostate cancer, which has metastasized to part of or the entire tumor population. This is significant because, with current treatment modalities, 10% of all men will be diagnosed with prostate cancer each year. Thus, patients who present with micrometastatic disease requiring systemic therapy from the ~120,000 men per year without micrometastatic disease, who require only local treatment.

Loss of function of metastasis suppressor gene(s) is an important event during the progression of a tumor cell from a nonmetastatic to a metastatic phenotype (5). Therefore, metastasis suppressor genes are potential markers to distinguish nonmetastatic and metastatic cancer cells. KAI1 is a metastasis suppressor gene located on human chromosome 11p11.2 (6). After transfer of the KAI1 gene into highly metastatic prostatic cancer cells (6) and mammary cancer cells (7), KAI1-expressing cancer cells are suppressed in metastatic ability, whereas their primary tumor growth is not affected. KAI1 is highly expressed in normal prostate; however, its expression is either not detectable or detected at a very low level in human prostatic cancer cell lines originally derived from distant metastases (6). These results suggest that decreased KAI1 expression is involved in the progression of human prostatic cancer to a metastatic state. Recently, it was reported that decreased mRNA expression of KAI1 correlates with poor prognosis in patients with non-small cell lung cancer (8).

If decreased expression of KAI1 is to be useful as a marker of metastatic ability of prostatic cancer cells, there must be a consistent decrease in its expression in metatstatic sites of prostatic cancer. In addition, if loss of KAI1 function is involved in the progression of cancer cells to a metastatic state, then primary cancers that vary in malignant potential should be heterogeneous, with some having no KAI1 expression, some having reduced expression, and some having normal levels of expression. The majority of metastases from primary tumors would be predicted to have no or low levels of KAI1 expression. Thus, KAI1 expression was evaluated in a series of primary prostatic cancers and metastases using immunohistochemical analysis. In addition, molecular analyses were performed on these samples to determine if mutation or allelic loss is involved in the dysregulation of the KAI1 gene during the progression of human prostatic cancer.

Materials and Methods

Immunohistochemical Analysis. For the immunohistochemical detection of KAI1 protein expression, the following tissues were used: (a) BPH tissue from 7 patients obtained by transurethral prostatectomy; (b) normal prostatic tissue from glands not containing cancer from 2 patients undergoing radical cystoprostatectomy for bladder cancer; (c) normal prostatic tissue from glands containing prostatic cancer from 45 patients undergoing radical prostatectomy for prostatic cancer; (d) prostatic tissue from 49 patients undergoing radical prostatectomy for prostatic cancer; (e) androgen-independent primary prostatic cancer tissue from 15 patients with metastatic prostatic cancer undergoing transurethral prostatectomy for relief of obstructive symptoms after androgen ablation therapy; and (f) metastatic prostatic cancer tissue from the pelvic lymph nodes of 10 untreated patients with metastatic cancer.

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4387
Six-μm frozen sections were cut on a cryostat and fixed with acetone for 10 min at 4°C and air-dried. All subsequent steps were performed at room temperature. The sections were incubated with methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. After treatment with PBS containing 5% normal rabbit serum for 30 min, the sections were incubated with purified mouse monoclonal anti-KAI1 antibody (IgG2a subtype) at a 0.4-μg/ml protein concentration in PBS for 2 h in a moist chamber. This mouse monoclonal antibody (C33) is mono-specific for KAI1 (6, 9–10) and was generously supplied by Dr. Osamu Yoshie, Shionogi Institute for Medical Science, Osaka, Japan. After treatment with biotinylated rabbit antimouse immunoglobulin (Research Genetics, Huntsville, AL) in PBS for 30 min, the sections were incubated with biotin-streptavidin peroxidase complex (Research Genetics) for 30 min, followed by incubation with a 3.3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide mixture. The sections were counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene, and mounted. As a negative control, nonimmunized purified mouse IgG2a (Sigma Chemical Co., St. Louis, MO) at 0.4 μg/ml was used instead of the anti-human KAI1 antibody.

Staining intensity was estimated as positive when it appeared to be similar to that of glandular cells of either BPH or normal prostatic epithelium also present within the section. The staining pattern of KAI1 expression was classified as abundant (51–100% of cells were positively stained), decreased (5–50% positively stained cells), or negative (0–4% positively stained cells). Statistical analysis was performed using the χ² test, with significance determined at the P < 0.05 level.

**Mutational Analysis.** For mutational analysis, metastatic prostatic cancer tissue from 10 different patients were used. Seven of the tumors were from lymph node metastases, two were from liver metastases, and one was from a subdermal metastasis. Genomic DNA was isolated according to standard procedures (11). KAI1 mutation was analyzed by using PCR-SSCP as described previously (12–13). Primers were end-labeled with [γ-32P]ATP (Amersham, Arlington Heights, IL). PCRs were carried out in a volume of 50 μl including 50 ng of genomic DNA, 0.125 pmol of each primer, 0.3125 nmol of each deoxynucleotide triphosphate, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 0.5 mM (exons 6, 8, and 9) or 1.5 mM (exons 1–5, 7, and 10), MgCl₂, and 0.0625 units Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) with 35 cycles each, consisting of denaturation at 94°C for 30 s, annealing at 57°C for 60 s, and a final incubation at 72°C for 5 min in a thermal cycler (M. J. Research, Watertown, MA). The PCR products were electrophoresed on a 0.5% polyacrylamide gel containing 5% glycerol, 45 mM Tris-borate buffer, and 4 mM EDTA at room temperature for 3–5 h, as described previously (12). The gel was then dried and autoradiographed at —80°C.

**Results**

Expression of KAI1 Protein in Normal, BPH, and Cancerous Prostatic Tissues. Eighty-three samples of human prostatic tissues were immunohistochemically stained with a monoclonal antibody to detect KAI1 protein (Table 1). In normal prostatic tissues, KAI1 protein is expressed in the epithelial compartment but not the stromal compartment. In the epithelial compartment, KAI1 protein was expressed on the plasma membranes of both basal and luminal glandular cells (Fig. 1A). Incubation with purified nonspecific mouse immunoglobulin IgG2a, which is the same isotype of the monoclonal antibody specific for KAI1, did not result in any positive staining of the normal epithelium of the prostate (Fig. 1B). Seven BPH samples were stained with KAI1 monoclonal antibody. As in normal prostate, a similar level of KAI1 expression was detected on the plasma membranes of epithelial cells in BPH tissue (Table 1).

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>No. of samples analyzed</th>
<th>Abundant</th>
<th>Decreased</th>
<th>Negative</th>
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<tr>
<td>BPH</td>
<td>7</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal prostatic tissue</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>from glands with no cancer</td>
<td>45</td>
<td>80</td>
<td>20</td>
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</tr>
<tr>
<td>from glands with cancer</td>
<td>49</td>
<td>27</td>
<td>57</td>
<td>16</td>
</tr>
<tr>
<td>PCa</td>
<td>15</td>
<td>0</td>
<td>32</td>
<td>60</td>
</tr>
<tr>
<td>From hormone untreated patients</td>
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<td>0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Metastatic prostatic cancer</td>
<td>2</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
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</table>

unrelated individuals (BIOS Lab, New Haven, CT), using PCR-SSCP as described above.

Microsatellite Analysis of Allelic Loss. DNA was isolated from 34 primary and 12 metastatic prostatic cancers as described previously (14). Primary prostatic cancers were obtained from radical prostatectomy specimens. Metastatic cancer samples were derived from pelvic lymph-node metastases. The polymorphic microsatellite marker D11S1344 is located in the KAI1 region, as defined previously (15). Primer sequences for D11S1344 are 5'-CCCT-TACA-GTGAGACC-3' (CA strand) and 5'-GGGCGTCTGAGGCCGG-3' AGAAGGAC-3' (downstream); and exon 10, 5'-GGGCGTCTGAGGCCGG-3' (upstream), 5'-TCCAGCTTCTGAGGCCGG-3' (downstream). The sequencing primers were the same as those used for PCR-SSCP. Primers were end-labeled with [γ-32P]ATP (Amersham, Arlington Heights, IL). PCRs were carried out in a volume of 50 μl including 50 ng of genomic DNA, 0.125 pmol of each primer, 0.3125 nmol of each deoxynucleotide triphosphate, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 0.5 mM (exons 6, 8, and 9) or 1.5 mM (exons 1–5, 7, and 10), MgCl₂, and 0.0625 units Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) with 35 cycles each, consisting of denaturation at 94°C for 30 s, annealing at 57°C for 60 s, and a final incubation at 72°C for 5 min in a thermal cycler (M. J. Research, Watertown, MA). The PCR products were electrophoresed on a 0.5% polyacrylamide gel containing 5% glycerol, 45 mM Tris-borate buffer, and 4 mM EDTA at room temperature for 3–5 h, as described previously (12). The gel was then dried and autoradiographed at —80°C. Abnormal bands detected by SSCP analysis were cut and eluted from the gel and amplified in 100 μl by PCR. The amplified PCR products were purified and sequenced using the cycle-sequencing method. The sequencing primers were the same as those used for PCR-SSCP. Primary sequences, which were derived from flanking intron sequences at least 30 bases away from the exon/intron junction so that aberrant splicing could be detected, are as follows: exon 1, 5'-GGGCGTCTGAGGCCGG-3' (upstream), 5'-TCTAGCTTGGCCCTGGAGG-3' (downstream); exon 2, 5'-AGGCGTCTGAGGCCGG-3' (upstream), 5'-CTGGGCGTCTGAGGCCGG-3' (downstream); and exon 3, 5'-GGGCGTCTGAGGCCGG-3' (upstream), 5'-GAGGAGAAGGCCGG-3' (downstream). In normal prostatic tissue, 78% had abundant KAI1 expression. Likewise, none of the metastatic cancer tissues surgically resected for urinary obstruction. In these androgen-independent primary prostatic cancers, only 8% still retained abundant KAI1 expression.
prostatic cancer deposits within lymph nodes from 10 hormonal untreated patients had abundant KAI1 expression (Table 1). Using \( \chi^2 \) analysis, the decrease in KAI1 protein expression is statistically significant \( (P < 0.05) \) in the progression from normal to localized to metastatic prostatic cancer.

**Mutation of KAI1 Gene in Prostatic Cancer Metastases.** The KAI1 gene consists of 11 exons, and its coding region starts from exon 3 and ends in exon 10.\(^4\) To amplify each exon of KAI1 by PCR, primers were designed from intron sequences flanking exons 1–9 and upstream of exon 10. The 3'-primer for exon 10 was derived from its sequence, which follows the stop codon. These primers were then used to amplify DNA from prostatic cancer metastases and the matched normal prostatic tissue from the same patient. The amplified PCR products were analyzed by SSCP for mutations. None of the 10 metastases showed a band shift in the SSCP assay.

**Allelic Loss of KAI1 in Prostatic Cancer Metastases.** During the study on KAI1 mutation, four polymorphisms were identified in KAI1 exons 7, 8, and 10. We then analyzed the heterozygosity of these polymorphic exons in DNA from 50 unrelated healthy individuals. The results are summarized in Table 2. The heterozygosity for each marker is 4, 36.5, 47.2, and 30.4, respectively. The combination of four markers results in an 80% heterozygosity for at least one allele. Allelic loss at the 4 markers in DNAs from the 10 pairs of normal prostatic tissue and metastasis from the same patient was analyzed using PCR-SSCP. Eight of the 10 metastases were informative for at least 1 marker. No allelic loss was found in any of the eight informative cases using PCR-SSCP. In an earlier study, allelic loss and imbalance were observed in 6 of 10 informative metastases from Japanese patients living in Japan using the polymorphic microsatellite marker D11S1344, which is located near the KAI1 locus (15). In the present study, the D11S1344 marker was analyzed for allelic loss in 34 primary tumors and 12 metastases from American patients. No allelic loss was found in any of these tumors. Thus, combining the SSCP and microsatellite analysis data, none of 54 informative primary cancers and metastases from American patients have allelic loss of KAI1.

**Discussion**

KAI1 is located on human chromosome 11p11.2 and is a metastasis suppressor gene, based on the fact that it can inhibit metastasis but not tumorigenesis when expressed in rat prostatic cancer cells (6). In the same study, it was also demonstrated that KAI1 expression is dramatically reduced in four human prostatic cancer cell lines derived from metastases. Recently, a significant correlation between the decreased expression of KAI1 mRNA and poor prognosis was reported for patients with non-small cell lung adenocarcinoma (8). To test if KAI1 is also involved in the progression of human prostatic cancer, KAI1 protein expression was analyzed in a series of normal, BPH, and

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malignant human prostatic tissues by immunohistochemical staining. KAI1 protein expression is detected in all normal prostatic tissue and is located on the plasma membranes of normal prostatic epithelial cells. In all the BPH tissues tested, similar or higher levels of KAI1 protein expression were detected on epithelial plasma membrane, indicating that in noncancerous neoplastic prostatic cells, KAI1 expression is maintained. The location of KAI1 protein on the membrane of epithelial cells suggests that KAI1 is involved in the interactions between cells and/or cells and their extracellular matrix. This interaction may mediate signaling between cells and their environment, affecting cell movements and differentiation. Indeed, in earlier studies, it was demonstrated that after gene transfer to induce the reexpression of KAI1 protein in highly metastatic rat prostatic cancer cells, these cells decreased their invasiveness and motility (6).

KAI1 protein expression was down-regulated in a subset of primary human prostatic cancers and all of the metastases examined. These results indicate that partial or complete loss of KAI1 function is associated with the progression of prostatic cancer to a metastatic state. Unfortunately, we have been unable to test whether there is a correlation between KAI1 protein expression and prostatic cancer-specific survival because of technical difficulties related to the KAI1 monoclonal antibodies currently available. The C33 monoclonal antibody used in the present study can only detect KAI1 protein in frozen tissue, but not in paraffin-embedded sections. Attempts to use a variety of antigen-retrieval protocols (e.g., heat, microwave, and/or enzymatic pretreatment) have all failed. We are now in the process of developing new antibodies that can detect KAI1 protein in paraffin-embedded tissue. Once available, such antibodies can be used to screen paraffin-embedded archival tissues to test the correlation between the level of KAI1 protein expression and tumor metastatic ability, survival rate, and so forth. These studies will validate whether KAI1 protein down-regulation is useful as a prognostic marker for prostatic cancer metastasis.

Besides down-regulation, gene mutation is another important mechanism for loss of function. To evaluate the role of KAI1 mutation in tumor progression, we analyzed 10 prostatic cancer metastases for mutations of the KAI1 gene. None of the 10 metastases had a mutation in the KAI1 gene. Therefore, down-regulation rather than mutation is a more common mechanism for dysregulation of the KAI1 gene in the progression of human prostatic cancers.

Allelic loss results in the reduction of gene dosage and thus may result in decreased expression. To test whether allelic loss could be a mechanism for the observed down-regulation of KAI1 protein in prostatic cancer, we analyzed metastases from patients with prostatic cancer by either PCR-SSCP (n = 8) or microsatellite assay (n = 46) using the polymorphic marker D11S1344, which is near the KAI1 locus. No allelic loss was found in 54 informative prostatic cancers and metastases. These results suggest that allelic loss of KAI1 is a rare event in the progression of prostatic cancer in Americans and that allelic loss is not responsible for the reduction of KAI1 protein expression in prostatic cancer metastasis in these American patients. In contrast, in Japanese prostatic cancer patients living in Japan, allelic loss or imbalance was found in 6 of 10 informative metastases (15). Whether the difference in KAI1 allelic loss is related to the racial difference in incidence and/or mortality between Japanese and Americans remains unclear, and studies are needed to compare this phenomenon in these different male populations.

At present, in situ hybridization studies using a biotinylated KAI1 probe are being performed to resolve whether the down-regulation of the KAI1 protein in prostatic cancer progression is due to down-regulation of KAI1 mRNA. Because several CpG-rich islands are present in the 5′ promoter region of the KAI1 gene, it is possible that methylation is involved in the transcriptional silencing of KAI1. This is also being tested.

References

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