Functional Evidence for a Metastasis Suppressor Gene for Rat Prostate Cancer within a 60-Kilobase Region on Human Chromosome 8p21-p12

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Abstract

We recently demonstrated that the human chromosome 8p21-p12 region encodes a metastasis suppressor gene for rat prostate cancer. The presence of this region suppresses the metastatic ability of rat prostate cancer cells (N. Nihei et al., Genes Chromosomes Cancer, 17: 260–268, 1996). To define further the region harboring the metastasis suppressor gene, a truncated human chromosome 8 containing this region was transferred into highly metastatic AT6.3 rat prostate cancer cells by microcell-mediated chromosome transfer. The region of human chromosome 8 retained in each microcell hybrid was determined by a PCR analysis with sequence-tagged site markers, and this analysis placed the metastasis suppressor gene in the interval between D8S2249 and D8S2244 on human chromosome 8p21-p12. One of the metastasis-suppressed microcell hybrids was used for construction of representative yeast/bacterial artificial chromosome (YAC/BAC) library covering the candidate region using a transformation-associated recombination technology (N. Kouprina et al., Genomics, 53: 21–28, 1998). The final contig corresponding to the candidate region was assembled by four YAC/BAC clones. Each clone was transfected into the AT6.3 cells, and the resultant transfectants were tested for their metastatic ability in athymic nude mice. Introduction of a 60-kb YAC/BAC clone resulted in significant suppression of the metastatic ability without suppression of the tumorigenicity. In contrast, other YAC/BAC clones in the contig had neither metastasis nor tumor suppressor activity. This demonstrates that the 60-kb fragment from the human chromosome 8p21-p12 region contains the metastasis suppressor gene for the AT6.3 cells. Frequent loss of heterozygosity of this region is detected in human prostate cancer, which suggests that our target metastasis suppressor gene may also play an important role in the progression of human prostate cancer.

Introduction

There is growing evidence that loss of function of metastasis suppressor genes plays an important role in cancer metastasis (1). To identify metastasis suppressor genes on human chromosomes, several groups have used the refined process of transferring individual human chromosomes into highly metastatic Dunning R-3327 rat prostate cancer cells by microcell-mediated chromosome transfer. In these studies, introduction of any one of the human chromosomes 2, 7, 8, 10, 11, 12, 16, and 17 into the highly metastatic rat prostate cancer cells resulted in suppression of the metastatic ability without suppression of the tumorigenicity (2–10). Using this model, our laboratory cloned KAI 1, a metastasis suppressor gene for prostate cancer, on human chromosome 11 (11). KAI 1 protein expression is consistently down-regulated during the progression of human prostate and other cancers (12, 13).

In the present study, we undertook a refinement of the region of the metastasis suppressor gene on human chromosome 8. In our previous study, the human chromosome 8p21-p12 region was found to encode the metastasis suppressor gene for rat prostate cancer (5). This region is the site of frequent LOH in numerous tumors (14–16) including prostate cancer (17–19). Specifically, several investigations have found that LOH at 8p21-p12 was associated with a more advanced clinical stage and invasive behavior (14–18), which suggested that our target metastasis suppressor gene for rat prostate cancer may play an important role in these human cancers.

To define further the region of the metastasis suppressor gene on human chromosome 8, a truncated human chromosome 8 with the metastasis suppressor activity that was generated with the initial irradiated microcell-mediated chromosome transfer was retransferred into the rat prostate cancer cells by microcell-mediated chromosome transfer. The resultant microcell hybrids were analyzed to determine which portion of human chromosome 8 suppressed the metastatic ability of the rat prostate cancer cells.

Materials and Methods

Cells. In the present study, a highly metastatic Dunning R-3327 AT6.3 subline was used. The development and characteristics of the AT6.3 subline were described previously (6). AT6.3 cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 0.4% penicillin-streptomycin (Life Technologies, Inc.) and 250 μM dexamethasone (Sigma Chemical Co., St. Louis, MO; standard medium) at 37°C with 5% CO2.

Microcell-mediated Chromosome Transfer. Microcell-mediated chromosome transfer was performed as described previously (20) using AT6.2-x8-7 (5) as the donor cells and AT6.3 rat prostate cancer cells as the recipient. The AT6.2-x8-7 cells contain a truncated human chromosome 8 with the metastasis suppressor activity (5). Human chromosome 8 containing AT6.3 microcell hybrids (AT6.3-8 series) were selected and maintained in the standard medium with 500 μg/ml of G418 (Life Technologies, Inc.).

PCR Analysis. PCR primers for STS markers on human chromosome 8 were used to identify the portion of human chromosome 8 retained in the various microcell hybrid clones. An extensive set of microsatellite markers on human chromosome 8 was used for the initial analysis (21), and the retained regions were refined by additional STS markers (22). The STS markers in the human chromosome 8p21-p12 region shown in Fig. 3B were as described and mapped by Mitsuda et al. (23). The reaction mixture was made up of the following components: 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5–2.0 mM MgCl2, 200 μM dNTPs, 0.5 μM primers, and 0.5 unit Taq Polymerase (Applied Biosystems, Foster City, CA) in a final volume of 10 μl. The PCR products were amplified for 35 cycles with annealing temperatures of 95°C, 56°C, and 72°C. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Support in part by Grant-in Aid 11307029 for Scientific Research (A) from Japan Society for the Promotion of Science (to T. I.).

Received 9/17/01; accepted 11/28/01.

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### Table 1  In vivo characteristics of AT6.3 and AT6.3-8 microcell hybrid clones and of BAC transfectants

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumorigenicity</th>
<th>Tumor volume(^b) (cm(^3))</th>
<th>No. of lung metastases/animal(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3 (parental)</td>
<td>9/9</td>
<td>3.2 ± 0.3(^d)</td>
<td>296 ± 17</td>
</tr>
<tr>
<td>AT6.3-8-15</td>
<td>5/5</td>
<td>3.5 ± 1.1</td>
<td>286 ± 21</td>
</tr>
<tr>
<td>AT6.3-8-25</td>
<td>5/5</td>
<td>5.3 ± 0.5</td>
<td>294 ± 15</td>
</tr>
<tr>
<td>AT6.3-8-26</td>
<td>5/5</td>
<td>4.0 ± 0.4</td>
<td>238 ± 36</td>
</tr>
<tr>
<td>AT6.3-8-22</td>
<td>5/5</td>
<td>5.4 ± 0.1</td>
<td>62 ± 26</td>
</tr>
<tr>
<td>AT6.3-8-28</td>
<td>5/5</td>
<td>5.4 ± 0.3</td>
<td>68 ± 25</td>
</tr>
<tr>
<td>AT6.3-8-33</td>
<td>8/8</td>
<td>3.3 ± 0.3</td>
<td>55 ± 15</td>
</tr>
<tr>
<td>AT6.3-1141-4</td>
<td>5/5</td>
<td>3.9 ± 0.1</td>
<td>223 ± 9</td>
</tr>
<tr>
<td>AT6.3-1141-4</td>
<td>5/5</td>
<td>4.4 ± 0.5</td>
<td>256 ± 17</td>
</tr>
<tr>
<td>AT6.3-920-5</td>
<td>5/5</td>
<td>3.7 ± 1.8</td>
<td>279 ± 24</td>
</tr>
<tr>
<td>AT6.3-920-6</td>
<td>5/5</td>
<td>3.4 ± 0.7</td>
<td>283 ± 47</td>
</tr>
<tr>
<td>AT6.3-920-8</td>
<td>5/5</td>
<td>6.9 ± 0.3</td>
<td>232 ± 52</td>
</tr>
<tr>
<td>AT6.3-920-5</td>
<td>5/5</td>
<td>7.1 ± 2.2</td>
<td>252 ± 38</td>
</tr>
<tr>
<td>AT6.3-920-6</td>
<td>5/5</td>
<td>4.4 ± 0.5</td>
<td>223 ± 42</td>
</tr>
<tr>
<td>AT6.3-920-8</td>
<td>5/5</td>
<td>5.5 ± 0.7</td>
<td>25 ± 16</td>
</tr>
<tr>
<td>AT6.3-127-6</td>
<td>6/6</td>
<td>4.0 ± 0.3</td>
<td>38 ± 11</td>
</tr>
<tr>
<td>AT6.3-127-9</td>
<td>8/8</td>
<td>4.7 ± 0.9</td>
<td>33 ± 10</td>
</tr>
</tbody>
</table>

\(^a\) Number of lung metastases/animal 5 weeks after the injections.
\(^b\) Number of tumor-bearing animals/number of animals used in the assay.
\(^d\) Mean ± SE.

Fig. 1. The regions of human chromosome arm 8q retained in AT6.3–8 microcell hybrids. +, marker retained; −, marker lost.

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**Results and Discussion**

Microcell-mediated chromosome transfer was used to introduce a truncated human chromosome 8 with the metastasis suppressor activity into highly metastatic AT6.3 rat prostate cancer cells. AT6.2-8x8–7 cells were used as the donor cells (5). Thirty-six AT6.3–8 cells were isolated. A cytogenetic analysis demonstrated that 6 of 36 clones conserved all of the rat chromosomes from the parental AT6.3 cells (data not shown). This conservation of rat chromosomes could minimize possible genetic changes within the rat genome that might influence the metastatic ability of the AT6.3 recipient cells. These six AT6.3–8 clones (AT6.3–8-15, -22, -25, -26, -28, and -33) were used for more detailed analyses.

To analyze the metastatic ability of the AT6.3–8 clones, the parental AT6.3 and the six microcell hybrid cells were injected s.c. into the nude mice. There were no significant differences in tumorigenicity between the parental AT6.3 and all of the six clones (Table 1).
AT6.3–8-22, -28, and -33 clones showed significant suppression of the metastatic ability, whereas AT6.3–8-15, -25, and -26 clones produced a high number of lung metastases in all of the inoculated animals (Table 1).

The portions of human chromosome 8 retained in the AT6.3–8 clones were determined by a PCR analysis with STS markers on human chromosome 8. The donor AT6.2-x8–7 clone contained a truncated human chromosome 8, consisting of two distinct regions derived from human chromosome arm 8p and 8q (Fig. 1). Additional STS markers were used to refine these two regions (Fig. 2 and 3A). The six AT6.3–8 clones retained all of the markers on the human chromosome arm 8q (Fig. 2). The metastasis-suppressed AT6.3–8-33 clone conserved all of the markers on human chromosome arm 8p (Fig. 3A). In contrast, other clones lacked several markers in this region. Specifically, AT6.3–8-22 contained the smallest derivative among the metastasis-suppressed clones. We used more densely spaced STS markers in the human chromosome 8p21-p12 region to refine further the region retained in AT6.3–8-22 (Fig. 3B). The metastasis-suppressed clones retained the region between D8S2249 and D8S2244, whereas all of the metastasis-unsuppressed clones lacked this region. These results suggest that the metastasis suppressor gene is located within the interval between D8S2249 and D8S2244 on human chromosome 8p21-p12 (Fig. 3B).

To construct a YAC/BAC contig covering this candidate region, a YAC/BAC library was constructed by TAR cloning. Human DNA fragments were selectively isolated as YAC/BAC clones with the neomycin resistance gene directly from the metastasis-suppressed AT6.3–8-33 clone using this method. The final contig was assembled by typing the YAC/BAC clones for all of the STS markers in this region, and the candidate region was covered by four YAC/BAC clones (Fig. 3B). The overlap of BAC 1141 and BAC 127 was confirmed by Alu profile characterization, which is based on hybridization of an Alu probe with TaqI-digested BAC DNA samples (data not shown). Each BAC clone was transfected into the AT6.3 cells, and

Fig. 3. Mapping of the human chromosome 8p21-p12 region. A, additional STS markers were used to refine the portion of human chromosome arm 8p retained in AT6.3–8 microcell hybrids. +, marker retained; –, marker lost. B, detailed PCR analysis with more densely spaced STS markers in the human chromosome 8p21-p12 region and BAC contig covering the candidate region for the metastasis suppressor gene. +, marker retained; –, marker lost. C, PCR products of BAC transfectants (D8S2249, D8S2248, and D8S2246).

369
the presence of the corresponding STS markers in the resultant trans- 
fecteds was confirmed by a PCR analysis (Fig. 3C). The BAC 
transfectants (AT6.3–1141, -127, -920, and -511) were tested for 
their metastatic ability in the spontaneous metastasis assay. In 
this study, introduction of 60-kb BAC 127 resulted in significant 
expression of the metastatic ability without suppression of the tumori- 
genicity, whereas other three BAC clones in the contig (BAC1141, 
920, and 511) had neither metastasis nor tumor suppressor activity 
(Table 1). This demonstrates that BAC 127 contains the metastasis 
suppressor gene for the AT6.3 rat prostate cancer cells.

The database search was performed to obtain the human genome 
sequence of the region between D8S2249 and D8S2247, in which 
BAC127 is included (26). Both markers are located in one BAC clone 
(GenBank no. AF252825), which indicates that this region can be 
covered by this BAC clone. However, complete sequence of this BAC 
clone is not available, which is needed to identify known genes and 
expressed sequence tags in this region.

In this study, we used refined STS markers that were generated 
recently. A PCR analysis with these markers demonstrated that the 
AT6.2-x–7 donor clone contained two distinct regions derived from 
human chromosome arm 8p and 8q (Fig. 1). The latter region had 
not been identified in our previous study. However, this region 
was retained in all of the AT6.3–8 clones including the metastasis- 
unsuppressed clones, which suggested that the metastasis suppressor 
gene is not located in this region. Furthermore, we identified a 
BAC clone with the metastasis suppressor activity in the human 
chromosome 8p21-p12 region. These results are consistent with 
our previous report demonstrating the presence of metastasis sup- 
spressor gene in the human chromosome 8p21-p12 region (5).

The short arm of human chromosome 8 is the site of frequent LOH 
in numerous tumors (14–16) including prostate cancer (17–19). Three 
regions, 8p22, 8p21, and 8p12, were defined as sites harboring tumor 
suppressor genes (27). Specifically, several investigations have found 
that LOH at 8p21-p12 was associated with a more advanced clinical 
and invasive behavior (14–18), which suggests that our target 
suppressor gene for rat prostate cancer may play an important role in 
these human cancers.

We used a functional positional cloning strategy to define 
the region harboring the metastasis suppressor gene, and localized it to 
a 60-kb cloned region. This functional approach allows identification of 
candidate BAC(s) in a critical region, and facilitate conventional 
positional cloning of a target gene. Efforts to identify the metastasis 
suppressor gene in the human chromosome 8p21-p12 region are 
currently underway.

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