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Advances in Brief

Functional Evidence for a Metastasis Suppressor Gene for Rat Prostate Cancer within a 60-Kilobase Region on Human Chromosome 8p21-p12[1]

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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[3] 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 nM dexamethasone (Sigma Chemical Co., St. Louis, MO; standard medium) at 37°C with 5% CO_2.

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 μ g/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. 3*B* 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 (pH8.3), 1.5–2.0 mM $MgCl_2$, 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

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[3] The abbreviations used are: LOH, loss of heterozygosity; STS, sequence-tagged site; TAR (cloning), transformation-associated recombination (cloning); BAC, bacterial artificial chromosome; YAC, yeast artificial chromosome.

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

Cell line	Tumorigenicity	Tumor volume[a] (cm^3)	No. of lung metastases/animal[b]
6.3 (parental)	9/9[c]	3.2 ± 0.3[d]	296 ± 17
AT6.3-8-15	5/5	3.5 ± 1.1	286 ± 21
AT6.3-8-25	5/5	5.3 ± 0.5	294 ± 15
AT6.3-8-26	5/5	4.0 ± 0.4	238 ± 36
AT6.3-8-22	5/5	5.4 ± 0.1	62 ± 26[e]
AT6.3-8-28	5/5	5.4 ± 0.3	68 ± 25[e]
AT6.3-8-33	8/8	3.3 ± 0.3	55 ± 15[e]
AT6.3-1141-1	5/5	3.9 ± 0.1	223 ± 9
AT6.3-1141-4	5/5	4.4 ± 0.5	256 ± 17
AT6.3-920-5	5/5	3.7 ± 1.8	279 ± 24
AT6.3-920-6	5/5	3.4 ± 0.7	283 ± 47
AT6.3-920-8	5/5	6.9 ± 0.3	232 ± 52
AT6.3-511-2	5/5	7.1 ± 2.2	252 ± 38
AT6.3-511-6	5/5	4.4 ± 0.5	223 ± 42
AT6.3-127-5	5/5	5.5 ± 0.7	25 ± 16[e]
AT6.3-127-6	6/6	4.0 ± 0.3	38 ± 11[e]
AT6.3-127-9	8/8	4.7 ± 0.9	33 ± 10[e]

[a] cm^3 5 weeks after injections.
[b] Number of lung metastases/animal 5 weeks after the injections.
[c] Number of tumor-bearing animals/number of animals used in the assay.
[d] Mean ± SE.
[e] $P < 0.05$ *versus* AT6.3 (parental).

STS marker	cM	AT6.2-x8-7 donor clone
8pter		
D8S504	0	-
D8S262	4.9	-
D8S561	8.4	-
D8S1706	10.5	-
D8S542	14.9	-
D8S520	19.3	-
D8S552	25.8	-
D8S511	29.5	-
D8S261	35.8	-
D8S258	40.3	-
D8S298	42.7	-
D8S1752	44.9	-
D8S1739	46.9	+
D8S1771	49.6	+
D8S1839	52.9	+
D8S1809	54.2	+
D8S1810	59.4	-
D8S1700	60.6	-
D8S1821	62.3	-
cen		
D8S538	66.9	-
D8S509	69.2	-
D8S507	75	-
D8S260	78.8	-
D8S553	81.5	-
D8S1785	82	-
D8S543	86.7	-
D8S279	90.2	-
D8S501	94.3	-
D8S275	97.2	-
D8S271	101	-
D8S270	102.1	-
D8S506	108.8	-
D8S521	114.8	-
D8S556	116.8	-
D8S555	122.6	+
D8S514	128.9	-
D8S1799	132.4	-
D8S266	135.1	-
D8S263	140.6	-
D8S284	142.7	-
D8S558	144.8	-
D8S256	147.6	-
D8S537	149.8	-
D8S274	153	-
D8S1741	161.5	-
D8S129	165	-
8qter		

Retained regions in the donor clone

Fig. 1. The regions of human chromosome 8 retained in the AT6.2-x8–7 donor clone. +, marker retained; −, marker lost. The genetic distances of the STS markers (*cM*) are indicated

AT6.3 -8 clones

	Donor	Unsuppressed			Suppressed		
cen → 8qter		15	25	26	28	33	22
CHLC.GATA68H01	-	-	-	-	-	-	-
AFMA082TG9	-	-	-	-	-	-	-
AFMB331YA9	+	+	+	+	+	+	+
D8S539	+	+	+	+	+	+	+
CHLC.GATA12F02	+	+	+	+	+	+	+
CHLC.GATA101F01	+	+	+	+	+	+	+
CHLC.GATA51F09	+	+	+	+	+	+	+
D8S555	+	+	+	+	+	+	+
CHLC.GATA64A06	-	-	-	-	-	-	-
D8S281	-	-	-	-	-	-	-

Fig. 2. The region of human chromosome arm 8q retained in AT6.3–8 microcell hybrids. +, marker retained; −, marker lost.

ranging from 55°C to 65°C by GeneAmp 9700 (Applied Biosystems), fractionated on 3% agarose gels, and visualized by ethidium bromide staining.

Spontaneous Metastasis Assay. To evaluate the metastatic ability, 5×10^5 cells were injected s.c. into the flank of 5-week-old male athymic nude mice. Tumor-bearing animals were sacrificed and scored for macroscopic lung metastases 5 weeks after the injections. Tumor volume at this end point was used as an index of tumorigenicity and was determined as described previously (24).

TAR Cloning, Characterization of YAC/BAC Clones, and Transfection. A human DNA was selectively cloned from one of the metastasis suppressed hybrids by TAR cloning using a vector containing *Alu* repeats as target sequences (25). A mixture of DNA prepared from the AT6.3–8-33 clone and linearized TAR circularizing vector pNKBAC39-Neo was presented to yeast spheroplasts, and transformants were selected on synthetic complete plates lacking histidine (25). The resultant YAC/BAC clones were transferred to *Escherichia coli* by electroporation. To construct a YAC/BAC contig covering the region between *D8S2249* and *D8S2244*, a PCR-based library screening was performed using the STS markers in this interval (*D8S2249*, *D8S2248*, *D8S1445*, *D8S2262*, *D8S2247*, *D8S2246*, *D8S2245*, *D8S339*, and *D8S2244*). *Alu* profile characterization was used to confirm the overlap of BAC 1141 and BAC 127 as described previously (25). The profiles were produced by hybridization of an *Alu* probe with *Taq*I-digested BAC DNA samples. The size of YAC/BAC clones was determined by transverse alternating field electrophoresis after a low-dose irradiation (25). Each YAC/BAC clone was transfected into the AT6.3 cells using Lipofectoamine Reagent (Life Technologies, Inc.). The resultant transfectants were selected and maintained in the standard medium with 500 μg/ml G418 (Life Technologies, Inc.). The transfectants were tested for their metastatic ability in the spontaneous metastasis assay.

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-x8–7 cells were used as the donor cells (5). Thirty-six AT6.3–8 clones 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).

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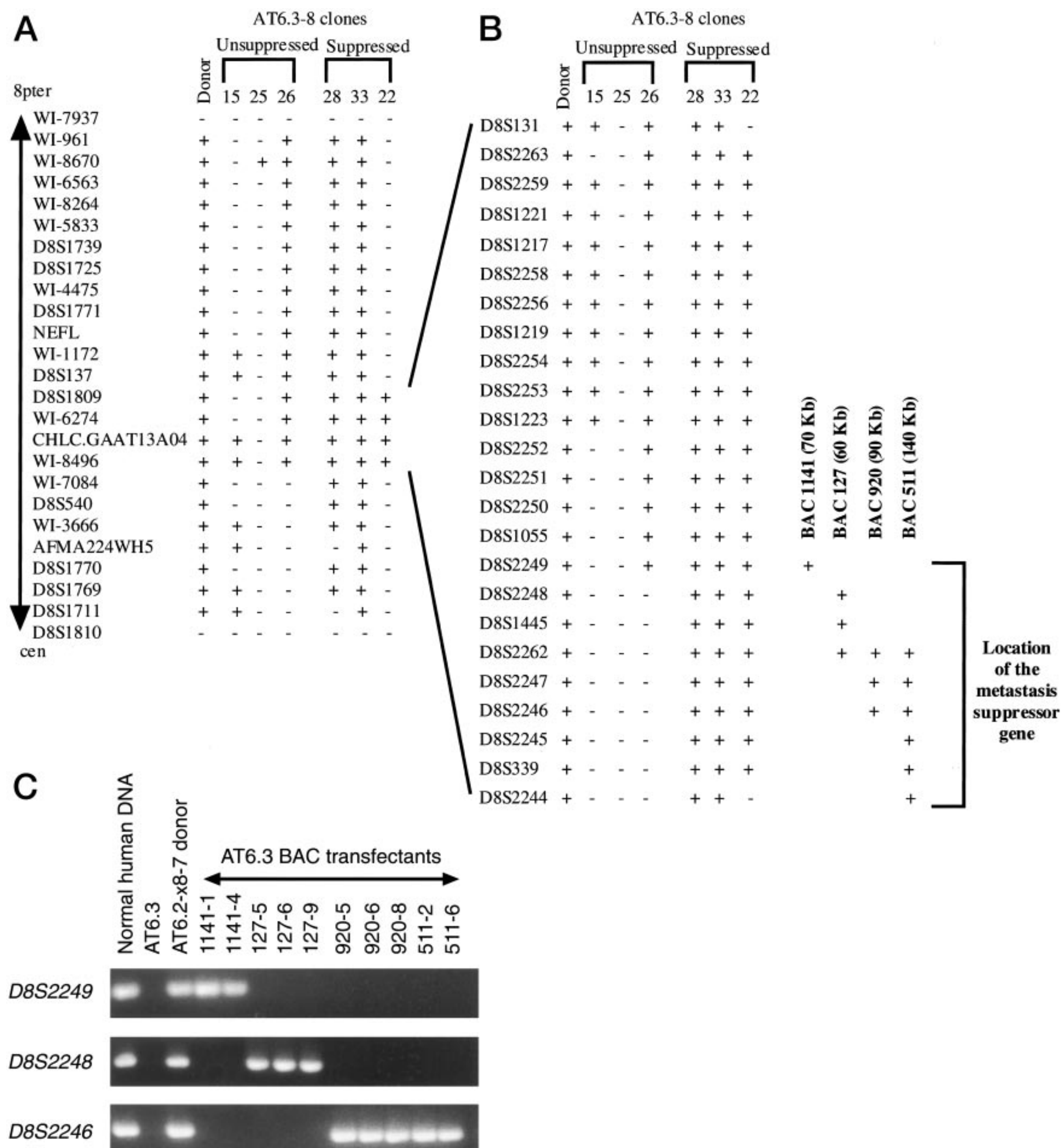

Fig. 3. Mapping of the human chromosome 8p21-p12 region. *A,* additional STS markers were used to refine the portion of human chromosome arm 8p retained in AT6.3–8 microcell hybrids. +, marker retained; −, marker lost. *B,* detailed PCR analysis with more densely spaced STS markers in the human chromosome 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*).

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 3*A*). 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. 3*A*). 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. 3*B*). 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. 3*B*).

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. 3*B*). The overlap of BAC 1141 and BAC 127 was confirmed by *Alu* profile characterization, which is based on hybridization of an *Alu* probe with *Taq*I-digested BAC DNA samples (data not shown). Each BAC clone was transfected into the AT6.3 cells, and

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the presence of the corresponding STS markers in the resultant transfectants was confirmed by a PCR analysis (Fig. 3*C*). The BAC transfectants (AT6.3–1141, -127, -920, and -511 series) were tested for their metastatic ability in the spontaneous metastasis assay. In this study, introduction of 60-kb BAC 127 resulted in significant suppression of the metastatic ability without suppression of the tumorigenicity, 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 BAC 127 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 sequenced 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-x8–7 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 suppressor 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 stage and invasive behavior (14–18), which suggests that our target metastasis 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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