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Advances in Brief

Suppression of Tumorigenicity of Human Prostate Cancer Cells by Introduction of Human Chromosome del(12)(q13)[1]

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Abstract

The introduction of normal chromosomes into tumor cells by microcell fusion-mediated transfer is a powerful technique to identify putative tumor suppressor genes. We have used this approach to independently transfer human chromosomes 3 and 12 into a human prostate cancer cell line, DU 145. We showed that while the extra copy of chromosome 3 had no effect on the *in vivo* tumorigenicity of these cells, microcell hybrids containing an introduced portion of chromosome 12 (12pter–12q13) exhibited complete suppression of tumorigenicity in athymic nude mice. The presence of a dual selectable marker facilitated the selection for cells having segregated del(12)(q13). Loss of this fragment in three different clones led to reexpression of the malignant phenotype. These results demonstrate that one or more genes on human chromosome 12 function as tumor suppressors of prostate carcinogenesis.

Introduction

Most of the evidence for the presence of tumor suppressor genes in human malignancies is derived from allelic loss detected by restriction fragment length polymorphism analysis. This has been especially well established in colorectal cancer, where allelic losses on chromosomes 5q, 17p, and 18q have been shown to correlate with neoplastic progression (1, 2). The 17p region corresponds to mutations in the previously cloned *TP53* gene (3), while new tumor suppressor genes *APC* (4) and *DCC* (5) have been cloned from the 5q and 18q regions, respectively. Allelotyping studies of prostate cancer tissues have shown that loss of heterozygosity occurs predominantly on chromosomes 8p, 10q, 16q, and 18q, implicating these regions as potential sites for tumor suppressor genes inactivated during prostate cancer development (6). Candidate tumor suppressor genes which correlate to these chromosomal loci have not yet been identified, although frequent loss of expression or loss of heterozygosity have been reported for the putative tumor suppressor gene *DCC* located in the 18q region (7).

Somatic cell hybridization offers an alternative approach for the identification of chromosomes which contain tumor suppressor genes. Fusion between a normal cell and a tumorigenic one often results in hybrids that are no longer tumorigenic. The nonrandom chromosome loss from nontumorigenic hybrids, resulting in reexpression of the malignant phenotype, can be used to identify chromosomes bearing tumor suppressor genes, leading to their eventual cloning (8). The major drawback of this methodology is that segregating hybrid cell populations are both heterogeneous and unstable. One approach to obtain better resolution involves the introduction of individual normal chromosomes in cancer cells by the process of microcell fusion-mediated transfer (9). We have developed a novel system which facilitates the isolation of segregant clones by tagging the chromosomes with the tgCMV/HyTK plasmid (10). This vector provides a means of selecting for the retention of the tagged chromosome with hygromycin B, while allowing negative selection of this chromosome through sensitivity to ganciclovir. Thus, the transfer of whole or fragmented chromosomes into cancer cells, resulting in suppression of their neoplastic phenotype, followed by the negative selection of the same chromosome to restore tumorigenicity, represents an effective strategy for the identification of chromosomal loci involved in tumor suppression.

Until now, only the introduction of a normal human chromosome 13, encoding the retinoblastoma gene, has resulted in significant although incomplete tumorigenic suppression of human prostate cancer cells (11). To identify tumor suppressor genes implicated in the pathogenesis of prostate cancer, we have performed microcell fusion to introduce human chromosomes into the DU 145 prostate cell line, hoping to implicate tumor suppressor loci which would otherwise be overlooked by allelotyping studies. We first isolated mouse/human microcell hybrids containing different tagged human chromosomes. This panel of microcell hybrids was then used to transfer specific tagged human chromosomes into G418-resistant DU 145 cells. Transfer of a portion of human chromosome 12, corresponding to 12pter–q13, resulted in complete tumor suppression in three different hybrids. The selective loss of this same portion of chromosome produced segregants which reverted to a tumorigenic phenotype. These results demonstrate that a putative tumor suppressor gene located on chromosome 12 is responsible for suppression of the malignant phenotype of prostate cancer cells.

Materials and Methods

Cell Lines and Culture Conditions. The DU 145 cell line (obtained from the American Type Culture Collection, Rockville, MD) and all other cells were maintained in monolayer culture at 37°C under 5% CO_2 in media supplemented with 10% FBS.[3] DU145-N19, obtained upon electroporation with pSV2neo plasmid, was grown in MEM containing 800 μg/ml G418 (GIBCO) and used as recipient for all microcell fusions. Human skin fibroblasts were established from foreskins and grown in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1 medium; PDI Bio Science Inc.). Cells (2×10^7) were electroporated with plasmid tgCMV/HyTK, which confers resistance to hygromycin B and sensitivity to ganciclovir.

Microcell-mediated Chromosome Transfer. All microcell fusions were performed as described by Fournier (12). The generation of the mouse/human hybrid panel will be described elsewhere. To transfer human chromosome 12, microcells were produced upon Colcemid treatment (0.06 μg/ml) of the mouse/human hybrid B78MC9 (containing a tagged portion of chromosome 12). These microcells were fused (90 s in 54% polyethylene glycol) to a monolayer of DU145-N19 cells. Microcell hybrids were selected in MEM-containing G418 (800 μg/ml) and hygromycin B (400 μg/ml). After 24 days, 13 hybrid clones were picked and expanded. Chromosome 3 was transferred

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[3] The abbreviations used are: FBS, fetal bovine serum; MEM, minimum essential medium; PCR, polymerase chain reaction; FISH, fluorescence *in situ* hybridization; SSC, standard saline citrate.

into the DU145-N19 cells by fusing microcells obtained from our tagged human skin fibroblasts population (90 s in 54% polyethylene glycol).

Cell Growth Studies. To determine the doubling time of the hybrids and segregants, 2×10^5 cells were seeded, and the cells were counted every day for 5 or 6 days. Doubling time was determined during log phase growth using values obtained from three experiments. To determine the anchorage-independent growth, trypsinized cells (5×10^4) were plated in agar (top layer: 3 ml, 0.3% noble agar (Difco); bottom layer: 3 ml 0.6% agar; both in MEM with 10% FBS) in 60-mm tissue culture dishes. Dishes were fed at weekly intervals with 1 to 2 ml of MEM supplemented with 10% FBS. Colonies of cells growing in soft agar were scored after 21 days in culture. The values are means of three dishes (13).

Nude Mice Injections. Suspensions of 5×10^6 cells in a volume of 0.3 ml of sterile saline were injected s.c. into 4–6 week-old male athymic *nu/nu* mice. All animals were monitored on a weekly basis up to 6 months after injection. Cells were considered to be nontumorigenic if no tumors were seen by 3 to 6 months after inoculation.

FISH. The *Alu9* probe (specific for human chromosome 12) was generated upon PCR amplification of B78MC9 DNA with four primers directed to the *Alu* consensus sequence (*Alu* primers 450, 153, 154, and 451; Ref. 14). The composition of the PCR reaction buffer was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, and 250 ng of consensus *Alu* primer. The reactions were carried out in a final volume of 50 μl with 1 unit of AmpliTaq DNA polymerase (Perkin Elmer Cetus) and 100 ng of genomic DNA under the following cycling conditions: 1 cycle at 94°C for 3 min, at 60°C for 1 min, and 72°C for 1 min; followed by 35 cycles at 92°C for 45 s, 60°C for 1 min, and 72°C for 1 min, with an increment of 6 s per cycle (35 cycles) and 1 cycle of 92°C for 45 s, 60°C for 1 min, and 72°C for 10 min. The four different PCR products were pooled and precipitated. The DNA was labeled by random priming with biotin-14-dCTP (BRL BioPrime DNA Labelling kit) and 2 μM [^{3}H]dTTP. Unincorporated nucleotides were eliminated by two ethanol precipitations. The level of biotin incorporation was determined by calculating the incorporation of [^{3}H]dTTP. Chromosomal spreads were prepared using standard techniques. After spreading, slide preparations were left overnight on a slide warmer at 55°C and stored at −20°C. Immediately before use, slide preparations were passed through an ethanol series (70–90–100%), denatured at 70°C in 70% formamide/2X SSC (1X SSC = 150 mM NaCl-15 mM sodium citrate, pH 7.0) for 2 min, and passed through another ethanol series (70–90–100%). The probe mixture (50% formamide, 10% dextran sulfate, 2X SSC, 90 to 100 ng of biotinylated probe, 1 to 3 μg of human Cot1 DNA (BRL), and 1 μg of salmon sperm DNA) was denatured at 75°C for 10 min and then preannealed at 37°C for 30 to 60 min. The hybridization mixture (10 μl) was applied to each slide preparation under a 22 x 22-mm coverslip, sealed with rubber cement, and incubated at 37°C for 16 to 20 h in a humidified chamber. The slides were washed at 45°C in 2X SSC, 50% formamide for 10 min, 2X SSC for 10 min, and 4X SSC-5% Triton-X 100 for 5 min. Avidin-fluorescein isothiocyanate detection was carried out according to Oncor protocols. The slides were mounted with propidium iodide/antifade mixture. Results were analyzed using an 09 filter combination on a Zeiss axioskop microscope equipped with epifluorescence.

Results

A panel of B78 mouse melanoma cells containing individual human chromosomes tagged with the tgCMV/HyTK vector was generated by fusion with microcells obtained from human skin fibroblasts.[4] One of the hybrids produced, B78MC9, contains a tagged portion of human chromosome 12 corresponding to 12pter–q13 [del(12)(q13)] and an untagged fragment of the same human chromosome. Microcell fusion was used to transfer chromosome del(12)(q13) from B78MC9 into DU145-N19, a G418^r derivative of the prostate cell line DU 145. The presence of tagged chromosomes in three independent microcell hybrids (DBM9–4, DBM9–7, and DBM9–10) was determined by Southern analysis using tgCMV/HyTK as probe (Fig. 1, Lanes *d*, *f*, and *h*). Karyotype analysis (data not shown) and *Alu*-PCR FISH using the *Alu*9 probe on DU145-N19 chromosome spreads demonstrated that DU145-N19 cells contain two apparently normal copies of chromosome 12 and a der(11), consisting of the long arm of chromosome 12 translocated to part of chromosome 11 (Fig. 2*A*). This particular chromosome marker is also present in DU 145 (data not shown). The hybridization patterns obtained for DBM9–4 (Fig. 2*B*) and DBM9–7 (Fig. 2*D*) microcell hybrids are similar to that of DU145-N19, except that an additional centromeric segment of chromosome 12 is present in all of the cells analyzed. Although the tgCMV/HyTK plasmid is present in the DBM9–10 hybrid (Fig.1, Lane *h*), the transferred portion of chromosome 12 could not be visualized by *Alu*-PCR FISH with the *Alu*9 probe, suggesting that DBM9–10 contains a very small portion of chromosome 12. Further analysis of this hybrid will help delineate the minimal portion of chromosome 12 capable of suppressing tumorigenicity. Even though chromosome del(12)(q13) might have been further fragmented during the transfer and has not been completely characterized in each DBM9 hybrid, we will continue to refer to the tagged transferred portions as del(12)(q13) since they were derived from that region.

All DBM9 hybrids were morphologically similar to the parental DU145-N19 cells, and the anchorage-dependent growth was decreased in only one (DBM9–7) of the three hybrids. The anchorage-independent growth, as assayed by growth in soft agar, was decreased in the hybrids, although no difference is apparent between the hybrids and their segregants (Table 1). Upon s.c. injection in athymic mice, the DBM9–4, DBM9–7, and DBM9–10 hybrid cells showed complete loss of their tumor-forming ability. Whereas the DU145-N19 cells showed tumor formation within 1 month after injection (6 tumors of 6 injection sites), no tumors were observed upon injection of the DBM9 hybrids (0 tumors of 13 injections), even 3 to 6 months after being inoculated (Table 1). These results indicate that the presence of the introduced portion of chromosome 12 eliminates the tumorigenic phenotype of DU145-N19 cells.

To demonstrate that the microcell fusion process in itself was not responsible for the suppression observed in the DU145-N19 cells, chromosome 3 was transferred into these cells by microcell fusion (see the DHM-5 hybrid, Table 1). The transfer of this chromosome (confirmed by karyotype analysis) did not alter the tumorigenic potential of DU145-N19 since 4 tumors of 4 injections appeared within 1 month following inoculation.

To confirm that the introduction of chromosome del(12)(q13) was the cause for the observed suppression (and to show that the suppressed phenotype was not due to the transfer of a mouse chromosome), the DBM9 hybrids were challenged with media containing 10 μM ganciclovir, which killed cells expressing the tgCMV/HyTK marker, thus allowing selection for cells having lost chromosome

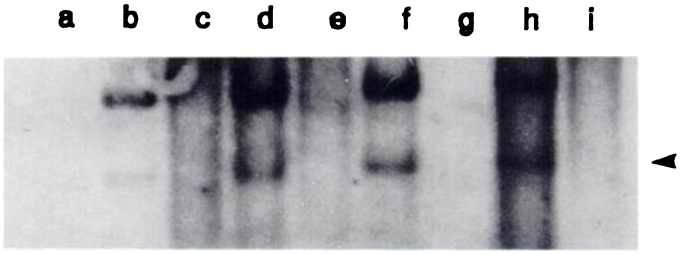

Fig. 1. Southern blot analysis of the hybrid cell lines and segregants. Genomic DNA (10 μg) from the following cells was digested with *Eco*RI and probed with the tgCMV/HyTK plasmid DNA. *a*, mouse B78; *b*, B78MC9; *c*, DU145-N19; *d*, DBM9–4; *e*, DBM9–4R; *f*, DBM9–7; *g*, DBM9–7R; *h*, DBM9–10; *i*, DBM9–10R. *Arrow*, the tgCMV/HyTK internal 1.85-kilobase *Eco*RI fragment.

[4] M. D. Speevak, N. G. Bérubé, I. J. McGowan-Jordan, S. D. Lupton, and M. Chevrette. Construction and analysis of microcell hybrids containing dual selectable tagged human chromosomes, submitted for publication.

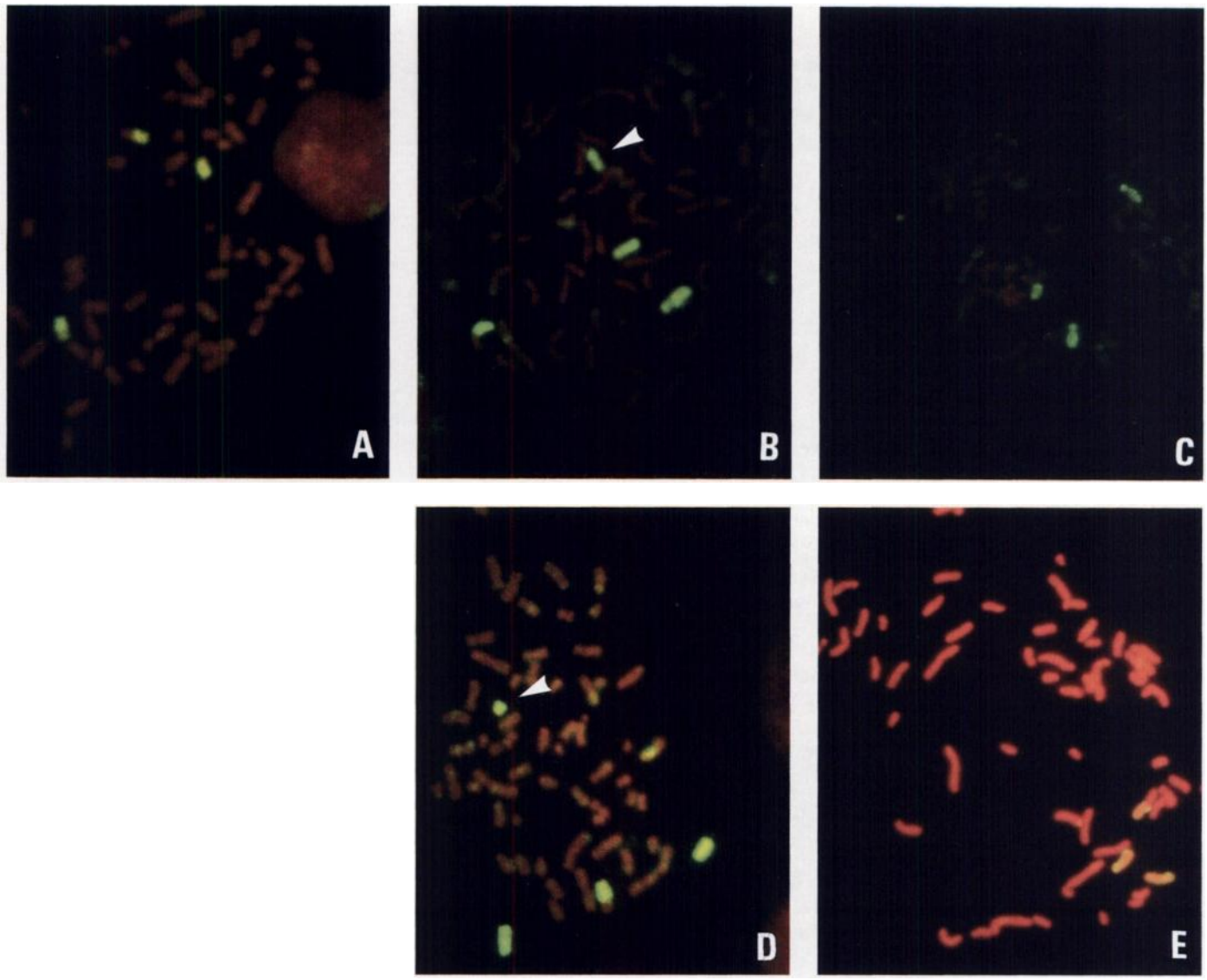

Fig. 2. Chromosome 12 FISH analysis of hybrid cells and segregants. *Alu*9 probe (specific for human chromosome 12) was hybridized to metaphase spreads of the following cells: *A*, DU145-N19; *B*, DBM9–4; *C*, DBM9–4R; *D*, DBM9–7; *E*, DBM9–7RTD. *B* and *D*, *arrows*, chromosome del(12)(q13) transferred by microcell fusion.

del(12)(q13). Three segregants obtained in this manner (DBM9–4R, DBM9–7R, and DBM9–10R) reverted to complete hygromycin sensitivity (Table 1). Southern blot analysis using tgCMV/HyTK probe (Fig. 1, *Lanes e, g,* and *i*) confirmed that the tag present in the DBM9 hybrids was selectively lost in the respective segregants. *Alu*-PCR FISH on the DBM9–4R segregant with the *Alu*9 probe demonstrated that the introduced chromosome del(12)(q13) was, in fact, lost in 100% of the cells analyzed (Fig. 2*C*). Similar results were obtained

Table 1 *Properties of parental and hybrid cells*

Cells	Growth in hygromycin B (400 μg/ml)	Doubling time (h)[a]	Growth in soft agar (%)[b]	Tumorigenicity[c]	Latency period (mo)	Presence/absence of tagged del(12)(q13)[d]
DU145-N19	–	35.3	4.28	6/6	<1	–
DBM9-4	+	35.4	0.03	0/5	6	+
DBM9-4R	–	31.5	0.53	4/4	<1	–
DBM9-7	+	43.3	1.43	0/4	6	+
DBM9-7R	–	50.5	1.57	3/5	<1	±
DBM9-7RTD[e]	–	33.0	ND[f]	ND	ND	–
DBM9-10	+	38.3	0.45	0/4	6	+
DBM9-10R	–	25.8	0.50	4/4	<1	–
DHM-5	+	N.D.	ND	4/4	<1	Introduced chromosome 3

[a] Determined during log phase of growth from triplicates.
[b] Number of colonies obtained divided by 50,000 cells plated, expressed as a percentage.
[c] Number of tumors formed per number of sites inoculated.
[d] As determined by Southern blot analysis and/or *Alu*-PCR FISH.
[e] Cells were resected from a tumor derived from DBM9-7R cells.
[f] ND, not determined.

for DBM9–7R, although 30% of the cells analyzed still retained the introduced chromosome del(12)(q13).

Unlike the DBM9 hybrids, the three segregants formed tumors (11 tumors of 13 sites inoculated) within 1 month after injection into nude mice (Table 1). One of the tumors, formed upon injection of DBM9–7R cells, was resected and cultured *in vitro*. Analysis of these tumor-derived cells (DBM9–7RTD) by *Alu*-PCR FISH using the *Alu*9 probe revealed that 100% of the cells analyzed had lost chromosome del(12)(q13), suggesting that the tumor originated from cells having lost the tagged chromosome. Therefore, selective loss of introduced chromosome del(12)(q13) in three distinct microcell hybrids restored the original tumorigenic potential of DU145-N19 cells, demonstrating that suppression of tumorigenicity was, in fact, caused by the introduction and retention of chromosome del(12)(q13).

Discussion

Some evidence suggests that tumor suppressor genes such as *RB1* and *TP53* may be involved in the progression of prostate cancer (15). Due to a splicing mutation, the *RB1* gene is inactivated in DU 145 prostate cancer cells. Transfer of a wild-type *RB1* gene into these cells by complementary DNA retroviral infection effectively suppresses their tumorigenicity (16). Similarly, the transfer of chromosome 13 containing a normal *RB1* allele markedly, but not completely, suppresses the tumorigenicity of DU 145 cells (11). Both alleles of the *TP53* gene are mutated in the three prostate cell lines PC-3, Tsu-PR1, and DU 145. Transfection of the wild-type *TP53* gene *versus* a mutated *TP53* gene into PC-3 and Tsu-PR1 results in reduced colony formation (17). Although these results suggest a role for *RB1* and *TP53* in the progression of prostate cancer, these suppressor genes are only implicated in a subset of prostatic tumors, thus indicating the involvement of other, as yet unidentified, tumor suppressor genes. Recently, expression of the putative tumor suppressor gene *DCC* was shown to be down-regulated in prostate cancer cells (7), supporting the idea that, as in colorectal cancer, numerous tumor suppressor loci are inactivated during prostate carcinogenesis. Not surprisingly, in humans, both types of cancer occur after a long incubation period, indicating that many events are necessary for the development of the full tumorigenic phenotype.

The data described here suggest the presence of yet another putative tumor suppressor locus, located on chromosome 12, which completely suppresses the tumorigenicity of DU 145 prostate cancer cells. Upon selection for the loss of chromosome del(12)(q13) in the suppressed hybrids, the cells regained their original tumor-forming ability, demonstrating that the presence or absence of the chromosome del(12)(q13) in the hybrids correlates with their tumorigenic phenotype. This dual selection confirms that the presence of a locus on del(12)(q13) is responsible for the tumor suppression. It also makes it unlikely that the effects on tumorigenicity in three independently obtained hybrids and the three corresponding revertants are due to the transfer and subsequent removal of an untagged mouse chromosome encoding a tumor suppressor gene.

The introduction of a tagged chromosome del(12)(q13) did not affect the morphology of the cells, nor did it alter their anchorage-dependent growth, except maybe in the DBM9–7 hybrid. The observation that the DBM9–4 and DBM9–10 hybrids have similar doubling times *in vitro* as the parental cells [as previously proposed (9)] suggests that *in vitro* growth behavior and tumorigenicity are under separate genetic control. A similar phenomenon has been observed upon transfer of chromosome 13 into DU 145 (11).

The suppressive effect of chromosome 12 on tumorigenicity in other tumor models has not yet been demonstrated. For example, microcell fusion has previously been used to transfer a human chromosome 12 in human fibrosarcoma cells (HT1080) without affecting tumorigenicity (18). Similarly, the transfer of chromosome del(12)(q13) into human teratocarcinoma cells PA-1, does not suppress their tumorigenic potential or change their morphology (data not shown), suggesting some level of specificity for prostate cancer cells. Cytogenetic and allelotyping studies have not yet linked chromosome 12 to prostate cancer. This could be explained if the inactivation in prostatic tumors is due to small deletions or point mutations rather than gross rearrangements. A more careful analysis as was recently done for the *FCC* locus (located on human chromosome 2) associated with hereditary nonpolyposis colorectal cancer (19) might help to implicate this chromosome 12 region in the development of human prostate cancer. Chromosome 12 aberrations detected by cytogenetic analyses have been reported in a small subset of cancers, *i.e.,* human germ cell tumors, acute myeloid leukemia, lipomas, and pleomorphic adenomas of the salivary gland (20). Such rearrangements may cause the inactivation of genes on chromosome 12 which are involved in the etiology of these tumors. Since DBM9–10 contains a very small portion of human chromosome 12, further analysis of this hybrid will help delineate the minimal portion of chromosome 12 capable of suppressing tumorigenicity.

The mechanism of tumor suppression by the locus (or loci) present on chromosome 12 is still unknown. It is interesting that it effectively suppresses tumorigenicity of the DU145-N19 cells, despite the fact that both *RB1* and *TP53* genes are also mutated in this cell line. This implies that the putative suppressor gene can compensate for the absence of two well-established tumor suppressors and suggests a role for the gene product in the same signal transduction pathways as p53 and pRb or in a parallel redundant pathway. It is also possible that a change in gene dosage rather than complementation of inactivated alleles is responsible for the loss of tumorigenic characteristics. Regardless of the mechanism underlying the tumor suppression, this report adds support to the hypothesis that compounded losses of tumor suppressor genes contribute to prostate carcinogenesis.

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