Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10877–10881, November 1992
Genetics

Definition of a tumor suppressor locus within human chromosome 3p21–p22

ANN MCNEILL KILLARY*†, M. ELIZABETH WOLF‡, TROY A. GIAMBERNARDI*, AND SUSAN L. NAYLOR‡

*Hematopathology Program, Division of Laboratory Medicine, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030; and
‡Department of Cellular and Structural Biology, The University of Texas Health Science Center, San Antonio, TX 78284

Communicated by Ruth Sager, July 9, 1992 (received for review February 14, 1992)

ABSTRACT **Cytogenetic abnormalities and high-frequency allele losses involving the short arm of human chromosome 3 have been identified in a variety of histologically different neoplasms. These findings suggest that a tumor-suppressor gene or genes may be located in the region of 3p14–p25, although there has been no definitive functional proof for the involvement of a particular region of 3p. We report a rapid genetic assay system that has allowed functional analysis of defined regions of 3p in the suppression of tumorigenicity *in vivo*. Interspecific microcell hybrids containing fragments of chromosome 3p were constructed and screened for tumorigenicity in athymic nude mice. Hybrid clones were obtained that showed a dramatic tumor suppression and contained a 2-megabase fragment of human chromosomal material encompassing the region 3p21 near the interface with 3p22. With these hybrid clones, we have defined a genetic locus at 3p21–p22 intimately involved in tumor suppression.**

Detailed cytogenetic analyses and loss of heterozygosity studies on a wide variety of neoplasms have identified key regions of the human genome that encode putative tumor-suppressor genes (1–6). With these kinds of approaches, human chromosome 3 has been implicated in the etiology of a number of diverse tumor types. High-frequency allele losses associated with small cell lung carcinoma (SCLC) in the region of 3p21–p23 have been observed by Naylor and other investigators (7–9). Other human tumors that show allele loss on 3p include non-SCLCs (10) and breast and testicular carcinomas (11–13). Renal cell carcinoma tumors have been found to harbor a more proximal deletion than SCLC spanning the region from 3p14–p21 (14). These studies suggested the involvement of a tumor-suppressor gene(s) on 3p; however, there was not definitive proof that a particular region on 3p contains a tumor-suppressor gene. We now provide direct functional proof that defines a tumor-suppressor locus at 3p21–p22.

A rapid genetic assay system was developed for analysis of defined regions of human chromosome 3 in suppression of tumorigenicity *in vivo*. Previous studies have shown that it is possible to complement the genetic defect in particular human cancers (which show high-frequency allele loss on a defined chromosome) by the introduction of a normal copy of that chromosome containing a putative tumor-suppressor gene (15–20). Studies such as these have demonstrated the functional involvement of genetic loci on human chromosome 11 in cervical carcinoma (15) and Wilm's tumor (16), human chromosome 6 in melanoma (17), human chromosomes 5 and 18 in colon carcinoma (18), and human chromosome 3p in renal cell carcinoma (19). Experimental strategies such as these are exceedingly difficult to use to define exact regions containing tumor-suppressor loci because of the karyotypic complexity and stability of intraspecific hybrids. Dowdy *et al.* (20) generated deletion hybrids for chromosome 11p and subsequently used these hybrids as donors for microcell fusion into a Wilm's tumor cell line to define the region involved in suppression (20). For our experiments, a normal human chromosome 3 was introduced into a highly malignant mouse cell line that responded to this human chromosome by exhibiting significant tumor suppression *in vivo*. The interspecific nature of the hybrid allowed rapid and unambiguous identification of the donor chromosome, delineation of the role of this chromosome in *in vivo* tumor suppression, and, finally, localization of defined regions of 3p encoding a tumor-suppressor locus.

MATERIALS AND METHODS

Cell Lines. Normal diploid foreskin fibroblasts (Hs27) were obtained from the American Type Culture Collection. The mouse fibrosarcoma cell line A9 (21) is deficient in hypoxanthine phosphoribosyltransferase (HPRT$^-$) and adenine phosphoribosyltransferase (APRT$^-$). Cell lines were free of mycoplasma as judged by Hoechst 33258 staining.

Electroporation. For electroporation experiments, the protocol of Potter and coworkers (22) was modified for normal diploid human fibroblasts and for transfection using a commercial Bio-Rad gene pulser. The procedure for electroporation of pSV2*neo* into Hs27 is described elsewhere (A.M.K., unpublished data).

Microcell-Mediated Chromosome Transfer. The protocol for human microcell-mediated chromosome transfer followed the method of McNeill and Brown as modified by Killary and Fournier (23, 24). Microcell hybrids were selected in Dulbecco's modified Eagle's medium/F-12 medium containing 10% fetal bovine serum, G418 (750 μg/ml), 10 μM ouabain, and gentamicin (50 μg/ml).

Cytogenetic Analysis. Microcell hybrids were characterized by sequential G-banding and G-11 staining analysis and by fluorescence *in situ* hybridization. For fluorescence *in situ* hybridization, human placental DNA was labeled with biotin by nick-translation and hybridized to metaphase spreads with fluorescein isothiocyanate conjugated to avidin (25).

PCR Analysis of Hybrid Clones. PCR amplification using chromosome-specific primers was performed as described by Theune *et al.* (26). The primers for *GLB1* were 5′-TGA TGA AAG CCT GTG CTT TGA G-3′ and 5′-AAA CAA TAA GGG AAC CGT CTG T-3′. Reaction conditions for *GLB1* were 2.0 mM $MgCl_2$ with an annealing temperature of 68°C. The primers for *ALAS1* were 5′-TGC AAC TTC TGC ACC ACC-3′ and 5′-GTT GAA CTT CTG GTA GAC-3′. Reaction conditions for *ALAS1* were 1.5 mM $MgCl_2$ with an annealing temperature of 55°C. Nomenclature for PCR primers is that used in the human genome data base.

***In Vivo* Assays.** Microcell hybrid and parental A9 cells were injected subcutaneously at 1×10^6 cells into each of five male

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Abbreviations: SCLC, small cell lung carcinoma; Mb, megabase(s).

†To whom reprint requests should be addressed.

athymic BALB/c *nu/nu* mice (4–6 weeks old). Tumors were measured biweekly. Tumors were excised, explanted into culture, and maintained without G418 for chromosome analysis.

RESULTS

Interspecific Hybrid Assay System. Monochromosomal hybrids were generated after electroporation of the dominant selectable marker pSV2*neo* into early passage human foreskin fibroblasts (Hs27). One human fibroblast clone, designated Hs27f, contained an intact human chromosome 3 marked with *neo* (A.M.K., unpublished data). Hs27f served as donor for microcell fusion (23, 24) of a *neo*-marked human chromosome 3 into mouse A9 cells. Initially, A9 was chosen as a recipient cell to isolate the *neo*-marked human chromosome 3. Once isolated in the mouse cell background, this marked chromosome could then be transferred into any malignant cell line exhibiting 3p losses. However, upon transfer of chromosome 3 into the fibrosarcoma background, the microcell hybrids exhibited a decreased growth rate and a reduced colony size and plating efficiency in soft agar (unpublished data). These results were interesting in that A9 is a highly malignant mouse fibrosarcoma cell line and was in fact used in the pioneering studies of Harris and Klein to determine the genetic basis for suppression of malignancy in somatic cell hybrids (for review, see refs. 27 and 28). Fusion of A9 with normal diploid lymphocytes resulted in hybrids that were suppressed for malignancy *in vivo* (29). For our experiments, we wanted to determine whether the phenotypic changes observed *in vitro* in A9 microcell hybrids containing human chromosome 3 correlated with a significant suppression of *in vivo* tumor growth.

Hybrid Characterization. Monochromosomal microcell hybrids were characterized cytogenetically by sequential G-banding/G-11 staining and fluorescence *in situ* hybridization using total human DNA as a probe. Hybrids were subsequently analyzed by PCR using 52 chromosome 3-specific, species-specific PCR primers. Two HA series hybrids [(HA(3)BB and HA(3)CC)] are monochromosomal for human chromosome 3; a third clone, HA(3)A, contains chromosomes 3 and 7 at high frequency as well as a small human chromosomal fragment translocated to mouse as detected by G-11 analysis. These clones were further characterized for growth *in vivo*.

Analysis of Microcell Hybrids in Nude Mice. For *in vivo* analysis, 1×10^6 hybrid and parental A9 cells were injected subcutaneously into each of five (4–6 weeks old) athymic nude mice. In addition to the three HA(3) hybrids used for these experiments, two other microcell hybrids that contained either a human chromosome X or a human chromosome 2 [HA(X)I and HA(2)A] were injected as controls. Also

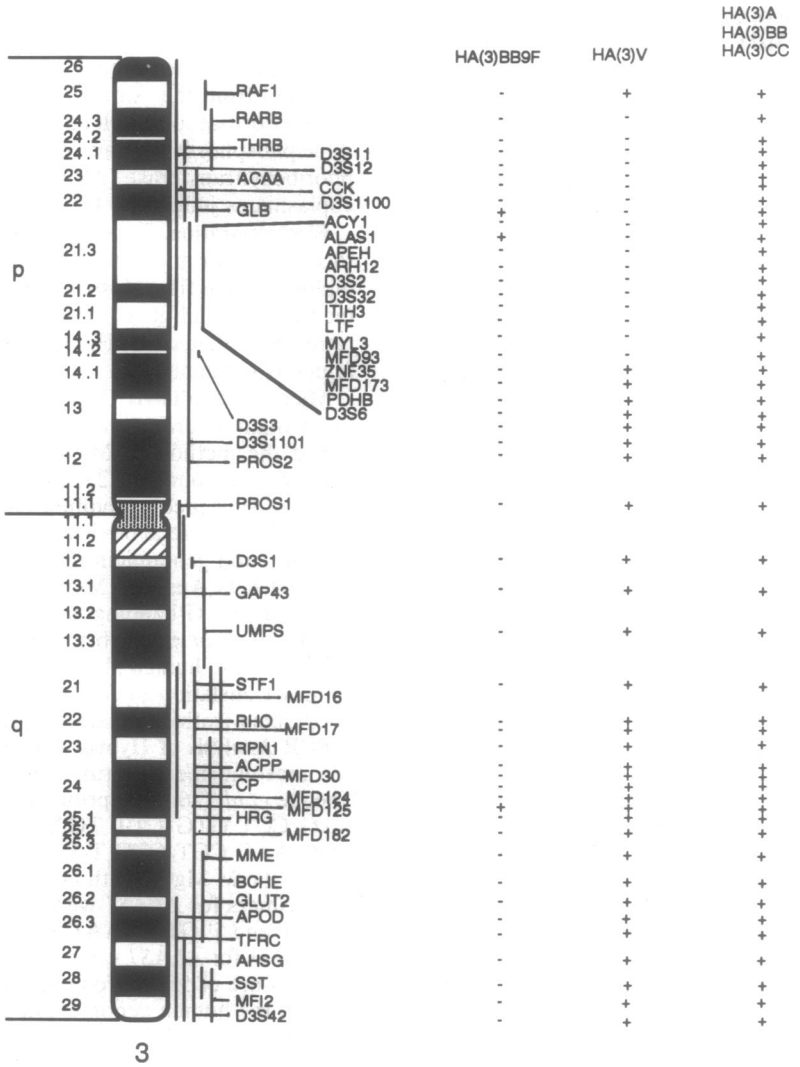

FIG. 1. Chromosome 3 map. Markers are a compilation of assigned loci reported in the Human Gene Mapping Workshop 11 (30). HA(3)A, HA(3)BB, and HA(3)CC contained all 52 markers and were suppressed for tumorigenicity *in vivo*. HA(3)V was not suppressed *in vivo* and contained a 3p14–25 deletion. HA(3)BB9F contained only 3 markers and was suppressed for tumorigenicity in nude mice.

a control for chromosome 3q, HA(3)V, was injected into nude mice. HA(3)V is a monochromosomal clone containing a deleted human chromosome 3. The chromosome 3q is intact in this hybrid both cytogenetically and by using 27 PCR primers specific for 3q; however, the 3p arm shows significant deletions (Fig. 1).

By 4 weeks, animals injected with A9 cells formed fibrosarcomas of an average wet weight of 1.9 g from four different experiments. The three HA(3) microcell hybrids showed a dramatic tumor suppression (Fig. 2). Although these results indicated significant tumor suppression, it was nevertheless incomplete, with all animals eventually developing small tumors. In contrast, the two monochromosomal clones HA(2)A and HA(X)I, and HA(3)V (the control for 3q), formed very large tumors by the end of 4 weeks (Fig. 2).

To determine whether the incomplete tumor suppression observed was consistent with chromosomal segregation from these interspecific hybrids *in vivo, in situ* hybridization studies were performed on hybrids immediately before and after injection. Results indicated that at the time of injection the introduced human chromosome was present at high frequency in the cell population (>90% of metaphases examined). After 4 weeks *in vivo*, HA(3)BB and HA(3)CC tumors were excised, explanted into culture, and examined for the presence of chromosome 3 both by cytogenetics and by PCR analysis. In all cases, the explanted cells had segregated chromosome 3 at high frequency with an average retention of 21%. HA(2)A and HA(X)I were also explanted in tissue culture and their chromosome content was examined. Significantly, human chromosomes were also lost from these interspecific hybrids, but to a lesser extent than HA(3)A, HA(3)BB, and HA(3)CC clones. Thus, the lack of tumor suppression observed in the control HA clones could not simply be a result of a more rapid loss of the introduced chromosome 2 or X *in vivo*.

Isolation of Fragment-Containing Subclones. Human–mouse interspecific hybrids eliminate human chromosomes with prolonged passage *in vitro* (31). Interspecific human microcell hybrids should also segregate the introduced human chromosome at some frequency that would be detectable by isolation of subclones grown without selective pressure in G418 and subsequently by assay of subclones for sensitivity or resistance to the antibiotic. Subclones sensitive to G418 would have lost either the entire chromosome 3 tagged with *neo* or the region of chromosome 3 carrying the selectable marker.

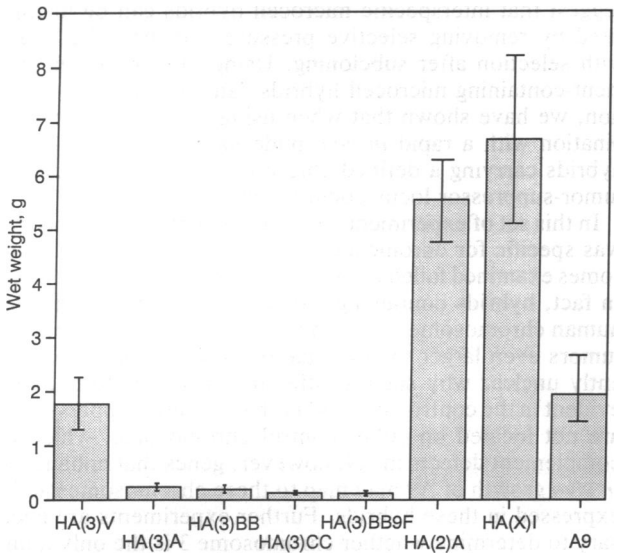

FIG. 2. Wet weights of tumors formed after injection of microcell hybrids and parental A9 cells in nude mice. Data are presented as average wet weight after 4 weeks *in vivo*. Hybrids described in Fig. 1 have been analyzed extensively in a large study either separately with A9 controls or in combinations with other hybrids. A9 is therefore represented as an average wet weight from 20 different animals.

Table 1. Tumor incidence in HA(3) clones and subclones 31 days postinjection

Cell line	Tumor volume,* ml	Tumor wet weight,* g	Animals with 1 cm^3 tumors†
HA(3)BB	1.05 ± 0.69	0.18 ± 0.07	2/5
HA(3)CC	0.58 ± 0.31	0.17 ± 0.18	1/5
HA(3)BB9C	0.36 ± 0.07	0.08 ± 0.05	0/5
HA(3)BB9E	0.91 ± 0.48	0.24 ± 0.06	1/5
HA(3)BB9F	0.34 ± 0.08	0.08 ± 0.08	0/5
HA(3)BB9X	1.19 ± 0.87	0.28 ± 0.12	2/5
HA(3)BB9Y	0.42 ± 0.16	0.17 ± 0.07	0/5
A9	4.36 ± 1.86	2.68 ± 0.42	5/5

*Mean ± SEM.
†No. of animals/total injected.

In an attempt to generate segregant clones that had lost *neo*, and therefore G418 resistance, HA(3)BB and HA(3)CC were subcloned in the absence of the antibiotic G418. Fifty-five subclones were originally isolated. Of these, 2 subclones, HA(3)BB9 and HA(3)BB21, were partially sensitive to G418. HA(3)BB9 was resubcloned to determine whether a pure population of sensitive cells could be obtained. Five of 12 subclones isolated were killed by G418. These 5 sensitive subclones were then screened for suppression of tumorigenicity in nude mice. All 5 subclones were greatly suppressed for tumor formation *in vivo* (Table 1). Subclone HA(3)BB9F (Fig. 3) was analyzed further by *in situ* hybridization with total human DNA used as a probe to detect the presence of any human chromosomal material. Results indicated that a small human chromosome 3 fragment of ≈2 megabases (Mb) is present in the HA(3)BB9F. The size estimate of the human fragment was based on fluorescence *in situ* hybridization analysis as well as the number of fragments detected by *Alu* PCR analysis (S.L.N., unpublished data). This human fragment is translocated to a murine chromosome (Fig. 3) and is present at very high frequency (>99%) in the cell population.

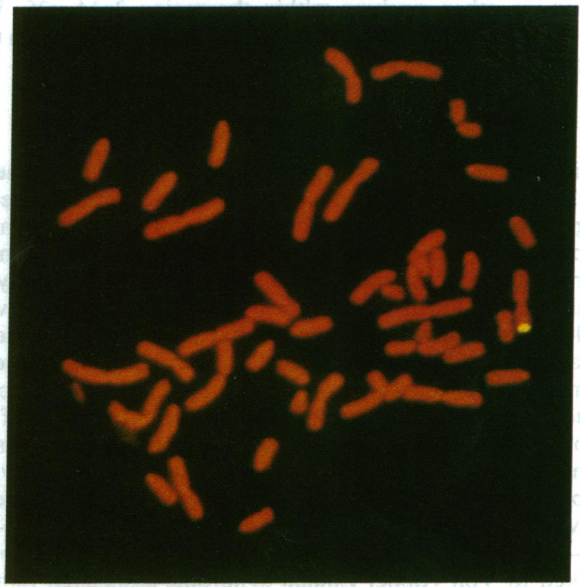

FIG. 3. Fluorescence *in situ* hybridization using total human DNA as a probe to detect the presence of a human chromosome 3p fragment in microcell hybrid subclone HA(3)BB9F.

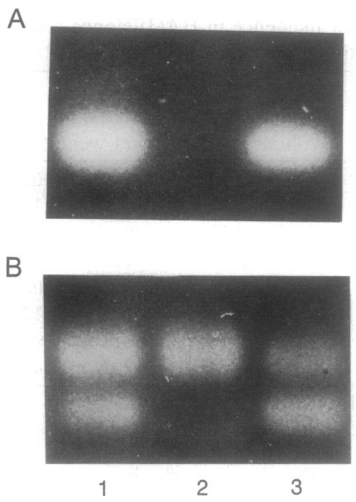

FIG. 4. PCR analysis of human *GLB1* (*A*) and *ALAS1* (*B*) genes. Amplification of HA(3)CC (chromosome 3 only), A9, and HA(3)BB9F with primers to *GLB1* and *ALAS1* produced human-specific fragments of 245 and 162 base pairs, respectively. Upper band in *B* is the mouse *ALAS1* gene.

When this clone was examined with chromosome 3 PCR markers (Fig. 4), the only 3p markers present of 27 examined were *ALAS1* (δ-aminolevulinate synthetase) and *GLB1* (β-galactosidase), both of which map to 3p21–p22 (Fig. 1). The order of markers in the 3p21 region is not completely known. However, data from one of our laboratories (S.L.N.) and others (30) would suggest that there are several markers between *GLB1* and *ALAS1*. Results suggest, then, that HA(3)BB9F contains a discontinuous segment of 3p.

Twenty-five PCR primers specific for 3q were also used to screen HA(3)BB9F. One marker *MFD125* (James Weber, personal communication), which maps to 3q21–3qter, was positive in the subclones tested. The 2-Mb fragment, therefore, contains at least one 3q marker. The HA(3)V control clone, however, which contained an intact 3q and significant 3p deletions (Fig. 1), made tumors comparable to parental A9 cells *in vivo* (Fig. 2). PCR analysis using 27 3p primers indicated that in HA(3)V the most distal p marker was preserved; however, *GLB1* and *ALAS1* were not detected, nor were other markers within the region 3p14–p25. We therefore conclude that the tumor suppressor gene lies in the region 3p21–22.

DISCUSSION

Using a rapid functional assay system, we define a genetic locus at 3p21–22 that encodes a tumor-suppressor gene. Significantly, the minimal region containing a tumor-suppressor gene has been narrowed to 2 Mb of human chromosome 3. Previous allele loss studies for a variety of malignancies localized the region from 3p14–p25. However, at least three different loci on 3p may be involved in genesis of a variety of human malignancies. The region implicated in familial renal cell carcinoma is 3p13–p21 (32–34). Recent studies suggest, however, that two distinct regions of 3p (3p13–14.3 and 3p21.3) show high-frequency allele loss in renal cell carcinoma (35). Studies focused on virtually all types of lung cancer, as well as breast and testicular cancer, have the highest degree of allele loss around 3p21–23 (7–10, 11–13). Finally, a more distal region at 3p24–p25 has been linked to Von Hippel Lindau syndrome-associated tumors (36). Our data then indicate that within the most consistent region of allele loss, a minimal region at 3p21–p22 has been identified that contains a functional tumor suppressor gene.

Although investigation into the function of tumor suppressor genes is still in its infancy, the best characterized examples of these kinds of genes function in a variety of different tissue types. p53 mutations are found in a host of different neoplasms; furthermore, inactivation or mutation of the retinoblastoma gene (*RB*) has been associated with a number of human malignancies including retinoblastoma, osteosarcoma, and SCLC, as well as cancers of the breast and bladder (for review, see ref. 37). The generality of involvement of a tumor-suppressor locus in many different tissues does not seem to be the case with the Wilm's tumor locus (*WT-1*). *WT-1* has a more limited pattern of expression in the kidney and a subset of hematopoietic cells and, therefore, may regulate growth and/or differentiation in a more tissue-specific manner (38). Allele loss studies would suggest that the gene or genes on 3p may be in the class of the general suppressor genes that are involved in a wide range of tumor types. Our data also suggest that chromosome 3 contains a general tumor-suppressor gene in that the introduction of human chromosome 3 can suppress tumors even in a mouse fibrosarcoma model system.

It is intriguing that the effect of human chromosome 3 on a mouse cell background would be so dramatic. One might speculate that the introduction of any human chromosome could retard the *in vitro* as well as *in vivo* growth of the hybrid cells to an extent that the latency period for tumor formation would also be affected. For this reason growth curves were generated for the hybrids used in this study (A.M.K., unpublished data). Although *in vitro* growth was retarded by the introduction of either chromosome 3 or chromosome X, the most dramatic decline in proliferative ability was clearly evident in HA(X)I. Quite the inverse effect was observed *in vivo*.

The utility of microcell hybrid systems for the definition of tumor-suppressor loci has been documented (15–20). The genetic assay system described here is unique in that an interspecific assay was developed that allowed both the definition of the locus as well as the rapid screening for the exact region involved in suppression. The success of this assay system is based on the relative ease with which microcell hybrids can be generated containing fragments of the introduced chromosome (39–41). Results from this study suggest that interspecific microcell hybrids can be manipulated by removing selective pressure and then challenging with selection after subcloning. Using this approach, fragment-containing microcell hybrids can be isolated. In addition, we have shown that when using this strategy in combination with a rapid *in vivo* nude mouse screen, valuable hybrids carrying a defined chromosomal region encoding a tumor-suppressor locus could be obtained.

In this set of experiments, the tumor suppression observed was specific for chromosome 3 because two other chromosomes examined failed to exert any tumor-suppressive effect. In fact, hybrids containing either human chromosome 2 or human chromosome X, when injected into nude mice, made tumors even larger than the parental A9 tumors. It is presently unclear why such a difference in tumor formation is evident in the control hybrids. Perhaps tumor-suppressor loci are not located on either control chromosome, which can complement defects in A9; however, genes that enhance the *in vivo* growth of A9 may map to these chromosomes and be expressed in these hybrids. Further experiments are necessary to determine whether chromosome 3 is the only human chromosome capable of tumor suppression in this model system or whether we may in fact be able to use this rapid assay to identify additional chromosomes and defined chromosomal regions containing general tumor-suppressor loci.

The hybrid clones generated in this study will be used to determine their functional significance in other human malignancies characterized by 3p losses as well as to provide the

starting material for isolation of the tumor-suppressor locus. Only then can the role of this tumor-suppressor gene be ascertained in malignancies such as lung cancer, which is the major cause of cancer mortality in the United States.

This research was supported by grants from the National Institutes of Health, the Mather Charitable Foundation, and the Kleberg Foundation.

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