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*Advances in Brief*

# A Common Region of Homozygous Deletion in Malignant Human Gliomas Lies between the *IFNα/ω* Gene Cluster and the *D9S171* Locus[1]

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## Abstract

**Deletions of the 9p-localized type-I interferon (IFN) genes and adjacent loci often occur during the development of malignant glioma. We have applied restriction fragment length polymorphism and microsatellite analysis to 12 loci covering this region of 9p and 3 loci on 9q in 74 human glial tumor tissues to define and further localize the smallest region of hemizygous or homozygous deletion common to the tumors. Three regions of homozygous deletion were evident among the panel of tumors; only one of these, however, residing between *D9S171* and the *IFNα/ω* gene cluster, was involved in multiple cases (13 glioblastomas). Hemizygous deletion of this same region was observed in an additional 27 tumors. In total these data indicate the frequent inactivation of a novel tumor suppressor gene residing adjacent to and centromeric of the type-I IFN genes in malignant gliomas.**

## Introduction

Cytogenetic and restriction fragment length polymorphism analyses of gliomas indicate consistent loss of genetic information at several specific genomic locations (1–4). One of the most common among the malignant gliomas [World Health Organization malignancy grades III and IV (5)] involves the loss of portions of the short arm of chromosome 9. Unlike losses of genetic information at some other locations, such as chromosome 10 where monosomy is common (1, 3), deletion mapping of 9p in tumors and cell lines has shown interstitial deletions and homozygous deletions limited to the region around the IFN[4] genes, suggesting that a TSG is localized in this area (2, 6, 7). In addition to gliomas, IFN gene deletions and/or 9p rearrangements have been described in a number of human malignancies, including lung carcinoma, mesothelioma, malignant melanoma, and leukemia (8–11), suggesting that inactivation of the same gene(s) may be important to the genetic etiology of several common cancers. In this study we have carried out an examination of loci in the region 9p21–22 in a series of 74 human glioma tissue samples. The findings limit the region of interest between the *D9S171* and the *IFNα/ω* loci and indicate a novel 9p TSG whose inactivation is associated with the development of malignant glioma.

## Materials and Methods

**Tumor Material.** Paired tumor and blood samples were collected, frozen immediately, and stored at −135°C for up to 4 years. All tumor pieces taken for molecular analysis were histologically evaluated. The 74 tumors consisted of 43 glioblastomas, 22 anaplastic astrocytomas, and 9 astrocytomas. All tumors were classified on the basis of the World Health Organization classification of tumors of the central nervous system (5).

**DNA Extraction and Analysis.** High molecular weight DNA from tumor pieces and blood was extracted as described previously (12). For Southern blotting 4 μg of DNA were digested with restriction enzymes using an automated laboratory work station (Biomek 1000; Beckman). The DNA was then electrophoresed on 0.8% agarose gels and alkali blotted to Hybond-N$^+$ membranes (Amersham). The membranes were hybridized with the DNA probes described below, labeled with [α-$^{32}$P]dCTP by random priming as previously described (12). Hybridized membranes were exposed to Storage Phosphor Screens (Molecular Dynamics, Sunnyvale, CA) and then scanned and analyzed on the Molecular Dynamics PhosphorImager.

**DNA Probes.** The following chromosome 9 loci were studied (probe name, restriction enzymes used, and origin): *D9S33* (CRI-1944; *Taq*I; Collaborative Research), *IFNB* (pBR13/FLO1; *Msp*I, *Ban*II; kindly supplied by Dr. A. von Gabain, Karolinska Institute, Stockholm, Sweden), *IFNA* [probe produced by PCR from genomic DNA; *Msp*I, *Taq*I, *Eco*RI; (6)], *D9S126* (p72-0.9; *Taq*I; kindly supplied by Dr. J. Fountain, Center for Cancer Research, Massachusetts Institute for Technology, Cambridge, MA), *D9S3* (DR6; *Hind*III; kindly supplied by Dr. N. C. Dracopoli), *D9S19* (CRI-1263; *Taq*I; ATCC), *GALT* (pc-DGALT; *Msp*I; ATCC), *D9S18* (pHHH220; *Taq*I; ATCC), *D9S29* (LAMP92; *Pvu*II; ATCC), *D9S16* (pMCOA12; *Taq*I; ATCC), *D9S30* (pMHZ21; *Pvu*II; ATCC).

**Densitometric Analysis.** Densitometric analysis of gene dosage was performed with the ImageQuant program on the Molecular Dynamics PhosphorImager. The variable number of tandem repeat probe pYNH24 detecting the anonymous locus *D2S44* was used as a reference for the assessment of gene copy number; this probe showed maintenance of constitutional heterozygosity in all tumor DNAs subjected to densitometry. The *LOI* hybridization signals were normalized against the signals measured at the control locus *CL* on the same blot according to the following formula:

$$\text{Number of alleles} = 2 \times N \times \frac{LOI_{(T)}}{LOI_{(B)}}$$

with

$$N = \frac{CL_{(B;A1)}/CL_{(T;A1)} + CL_{(B;A2)}/CL_{(T;A2)}}{2}$$

where *T* represents tumor, *B* represents blood, and *A*1 and *A*2 are larger and smaller alleles, respectively.

**PCR Amplification.** The following dinucleotide repeat sequence loci were studied (primer definition): *D9S43* (Mfd14CA/Mfd14GT) (13); *D9S169* (AFM 164 xg7) (14); *D9S171* (AFM 186 xc3) (14); *D9S199* (C101) (15); *D19S49* (Mfd11CA/Mfd11GT) (13); *D17S250* (Mfd15CA/Mfd15GT) (13); *D19S47* (Mfd9CA/Mfd9GT) (13). PCR was performed in 10-μl reaction volumes with 10 mM Tris-HCl, pH 8.3-50 mM KCl-1.5 mM $MgCl_2$-5 μM each dATP, dCTP, dGTP, and dTTP-0.5 μCi [α-$^{33}$P]dATP-0.5 μM each primer-100 ng genomic

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[4] The abbreviations used are: IFN, interferon; LOH, loss of heterozygosity; TSG, tumor suppressor gene; ATCC, American Type Culture Collection; *LOI*, locus of interest; *CL*, control locus; PCR, polymerase chain reaction.



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DNA-0.5–1.0 units Taq polymerase (Perkin Elmer). Eighteen to 20 cycles of amplification were carried out at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were electrophoresed on a 6% nondenaturing polyacrylamide gel at approximately 80 W, dried, and exposed to a Storage Phosphor Screen.

## Results

Losses of genetic material from chromosome 9 (Figs. 1 and 2) were seen in 48 (65%) of the 74 tumors. All instances of loss involved loci on 9p, while 4 of these (8%) also involved 9q loci. Among the glioblastomas, losses of genetic material from 9p were seen in 31 of 43 informative cases (72%), while losses of genetic material from 9q were limited to 4 of 21 informative cases (19%). The anaplastic astrocytomas showed an almost identical incidence of loss of genetic material from 9p: 16 of 22 informative cases (73%). The astrocytomas showed LOH on 9p in one of 9 (11%) informative cases. There were no losses of genetic information from 9q in either the astrocytomas or the anaplastic astrocytomas. While losses from 9p, in general, were similar in incidence among the glioblastomas and anaplastic astrocytomas, homozygous deletions were only found among the glioblastomas with 15 of 43 informative cases (35%) showing such alterations (Fig. 3).

When the incidence of loss of one allele (hemizygous deletion) at the various loci was tabulated for the anaplastic astrocytomas and glioblastomas, the findings showed an increase from the most centromeric locus studied, the *D9S18* locus (22%), to the *D9S126* locus (44%) and the *IFNα/ω* locus (43%); loci telomeric of the *IFNα/ω* gene cluster showed a decreasing incidence of deletion. The cases with homozygous deletions (all glioblastomas) collectively showed an increase in the incidence of homozygous deletion of loci from 0% at *D9S18* to 10 of 43 (23%) at the *IFNα/ω* locus, with the more telomeric loci showing decreasing incidences, reaching 0% at *D9S33*.

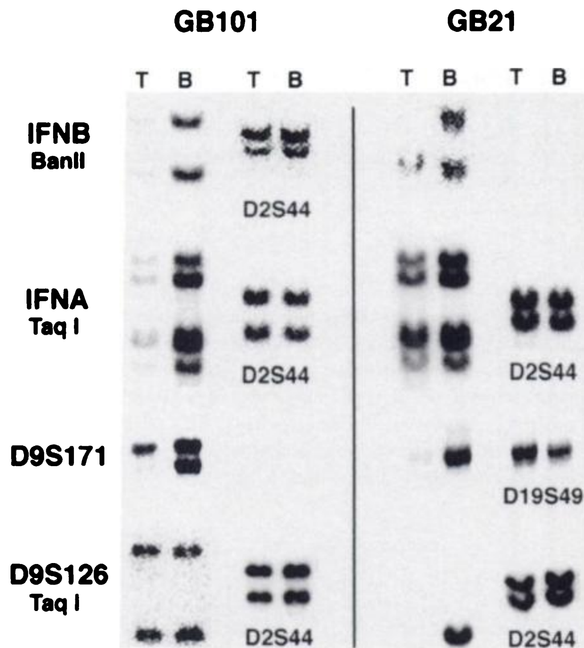


Fig. 1. Loci between *D9S126* and *IFNβ* studied by Southern blot (D9S126, IFNα, IFNβ) and microsatellite (D9S171) analysis are shown for two glioblastoma cases (GB101 and GB21). To assess loading of DNA, control hybridizations of the Southern blots at the highly informative *D2S44* locus and microsatellite analysis at the *D19S49* locus are shown to confirm homozygous deletion. T, tumor DNA; B, constitutional WBC DNA. The restriction enzyme used is noted under the locus designation. Case GB101 is an example of a case which is informative at *D9S126, D9S171,* and *IFNβ*. The tumor retains both alleles at the *D9S126* locus, shows LOH at *D9S171,* and loss of both alleles at *IFNα* and *IFNβ* (compare with *D2S44* control hybridization). This tumor also showed LOH at *D9S33* but retention of both alleles at *D9S199,* loci which have been mapped telomeric to *IFNβ* (for summary see Fig. 3). Case GB 21 shows another pattern of loss with deletion of both alleles in the tumor at the *D9S126* and *D9S171* loci but evidence for loss of only one allele at the *IFNβ* and *IFNα* loci. Thus, the common region for loss of both alleles in these cases is between the *IFNα* and the *D9S171* loci.

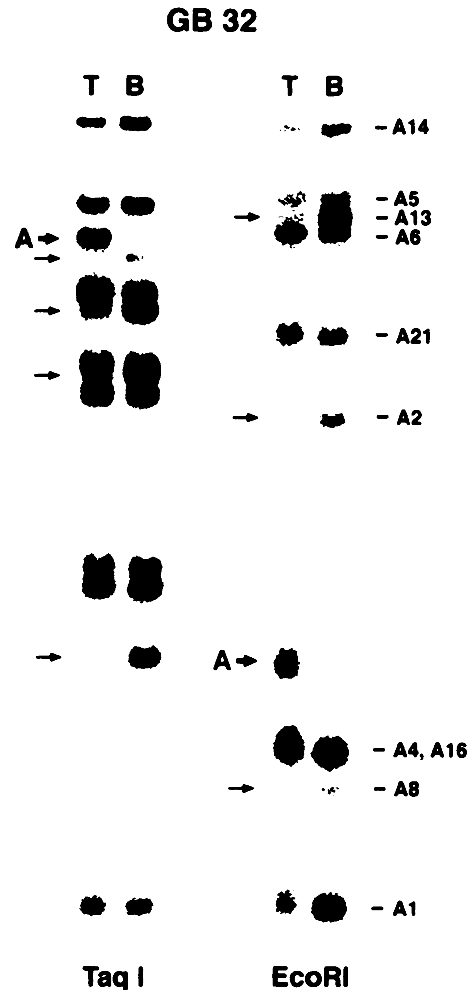


Fig. 2. Southern blot analysis of the *IFNα* locus in case GB32. T, tumor DNA; B, constitutional WBC DNA. Note the total loss of some of the *IFNα* normal bands (*small arrows*) with the appearance of aberrant bands (*A, large arrows*) in the tumor DNA. This tumor shows a homozygous deletion only at this locus (Fig. 3). The bands lost correspond to the *IFNα* genes which are located most centromerically within the cluster (16).

Because the *D9S171* locus was clearly located in the critical region of this study (Fig. 3), we carried out a control experiment to confirm that the PCR amplification conditions we used for this microsatellite could be evaluated quantitatively (Fig. 4). In this experiment two distinct (different patients) DNAs were mixed in varying proportions and added as template to a PCR mixture containing two primer pairs (D9S171 and D19S49). The signal intensity from each set of alleles was proportional to the amount of the corresponding template (Fig. 4). Based on these results and as an additional control of the PCR-based analysis, an extra primer pair was included in the reaction when homozygous deletions were suspected at a locus, to document the presence of genomic DNA and to permit a quantitative assessment (Fig. 1).

If we consider the glioblastomas individually (Fig. 3), we find that of the 31 tumors showing losses of alleles on 9p, 15 had homozygous deletions involving the adjacent loci *D9S171* and *IFNα/ω*. Tumors GB18 and GB97 showed loss of both alleles at the *D9S171* and *IFNα/ω* loci. GB97 lost, in addition, both alleles at the adjacent loci *D9S126* (centromeric) and *IFNβ* (telomeric), while GB18 showed retention of both alleles at *IFNβ* and loss of one allele at *D9S126,* limiting the area homozygously deleted to that around and including the *D9S171* and *IFNα/ω* loci.



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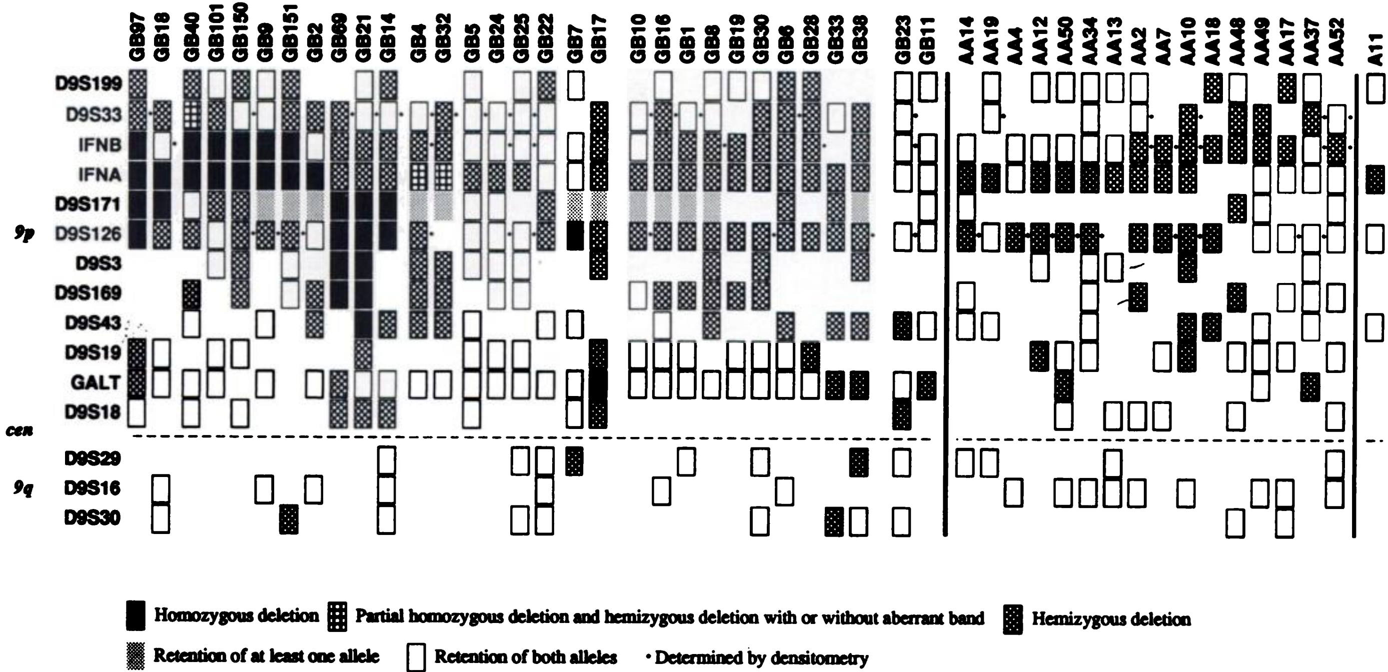


Fig. 3. Summary of the status of alleles at all loci studied in all cases in the series which showed loss of alleles at any chromosome 9 locus. Loci for which no data are given indicate loci where the case was not informative and where quantitative analysis was not carried out to determine the state of the alleles. *cen*, centromere.

The common region of homozygous deletion was further limited on the centromeric side by the tumors GB40, GB101 (Fig. 1), GB150, GB9, GB151, and GB2. All showed homozygous deletions of the *IFNα/ω* gene cluster with either retention of one or both alleles at *D9S171*.

The tumors GB69, GB21 (Fig. 1), and GB14 limited the extent of the common area of homozygous deletion telomerically. All three tumors showed homozygous deletions at the *D9S171* locus but showed retention of one allele at the *IFNα/ω* locus and, thereby, limited the telomeric ends of their homozygous deletions to the region between the *D9S171* and *IFNα/ω* loci.

The two tumors GB4 and GB32 (Fig. 2) showed the smallest region of homozygous deletion. Densitometric analysis revealed the complete loss of one allele at the *IFNα/ω* locus and, in addition, loss of part of the other allele and the appearance of an aberrant band. For both of these tumors the region of partial homozygous deletion involved the centromeric side of the *IFNα/ω* gene cluster (16). One allele was retained in each tumor at the *IFNβ* locus and, although the *D9S171* locus was uninformative in these two cases, there was no evidence of loss of both alleles.

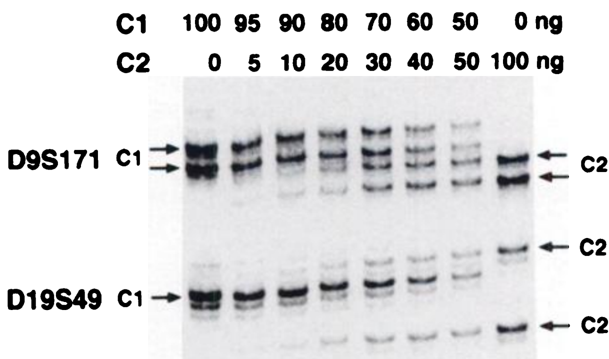


Fig. 4. Simultaneous microsatellite analysis at the *D9S171* and *D19S49* loci (see "Materials and Methods") to demonstrate that the method is quantitative. The template is made up of varying proportions (detailed above each slot) of constitutional genomic DNA from two individuals (C1 and C2). The autoradiographic signal from the alleles was analyzed by ImageQuant (PhosphorImager) and shown to be directly related to the amount of template for each allele present in the PCR.

The tumors with loss of single alleles at loci on 9p, although generally affecting a larger area than the homozygous losses, showed patterns of loss implicating the same region as being targeted for deletion (*i.e.*, between *D9S171* and *IFNα/ω*). Examples of this include cases GB5 and GB24, which showed loss of one allele only at the *IFNα/ω* locus, and GB22, which showed retention of both alleles at the *IFNα/ω* locus but losses of an allele at both the more centromeric loci *D9S171* and *D9S126*.

There were, however, a number of cases which showed patterns of loss which target other regions. Among the glioblastomas, two cases (GB7 and GB17) showed homozygous deletions which did not encompass the common region of loss described above. The region of homozygous deletion in GB7 was limited at its telomeric end by *D9S171*. This region showed LOH in many of the cases, and distinct interstitial deletions involving it occurred in tumors GB40 and AA14. Another distinct region of homozygous deletion which was bordered centromerically by the *D9S18* locus and telomerically by the *D9S19* locus was evident in GB17. This same region also was specifically involved in an interstitial deletion in tumor AA50 and possibly in GB11 and AA37 as well.

When chromosomal mechanisms are considered and the chromosome 9 molecular data (Fig. 3) are interpreted in the simplest terms, it is striking that the patterns of loss in the 48 tumors imply interstitial deletions of 9p in the majority of cases. Among the 31 glioblastomas the pattern of loss implies 15 tumors with one interstitial deletion, 9 tumors with two interstitial deletions, and 2 tumors with three interstitial deletions each. Seven of the 16 anaplastic astrocytomas and the single astrocytoma showed patterns of allele loss compatible with single interstitial deletions, with an additional two anaplastic astrocytomas showing losses compatible with two interstitial deletions each. These interstitial deletions occurred alone or together with patterns consistent with terminal deletions or monosomy.



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## Discussion

Homozygous deletions have proven invaluable in the identification of tumor suppressor loci, including WT1, RB1, and DCC (17–19). The frequent homozygous deletion of the type-I IFN genes in gliomas and glioma cell lines has motivated the identification of the genetic target associated with this alteration of chromosome 9p. The results of a previous study of glioma cell lines could be interpreted as supporting the notion that the IFN genes themselves are the target, since the majority (12 of 14) of instances of homozygous deletions involved the entire *IFNα/ω* gene cluster. However, the results of this paper suggest the possibility of a novel gene located between the *D9S126* locus and the type-I IFN genes. While the type-I IFN genes are still among the loci showing most frequent loss of alleles in this study, the present results do not point to the IFN genes being exclusively targeted. The region between *D9S171* and the *IFNα/ω* locus contains the sequences most often lost, implying the presence of an as yet unidentified TSG(s).

This region is included in the 9p zones identified as being specifically targeted in recent studies of lung cancer, malignant mesothelioma, malignant melanoma, and leukemia (8–11). Thus, it seems likely that the region contains a gene(s) or sequences which may be involved in many different human neoplasms.

The complexity of the rearrangements of the short arm of chromosome 9 and the infrequent occurrence of simple monosomy or loss of 9p contrast with the findings in gliomas for other chromosomes in which simple loss of the whole arm or monosomy is a common occurrence. Here, the simplest interpretation of the data suggests that 82% of the breakpoints identified on the chromosome are involved in interstitial deletions. The clarification of the reasons for this will require extensive study, but a simple explanation might be that many genes essential for survival of these cells are present on chromosome 9. This possibility as well as the identification of a novel TSG which resides between the *D9S171* and *IFNα/ω* loci will be addressed by the continued physical and deletion mapping of this critical region of 9p.

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