Homologous Deletions of 9p21 in Primary Human Bladder Tumors Detected by Comparative Multiplex Polymerase Chain Reaction

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Abstract
Deletion mapping studies of primary bladder tumors have identified nonoverlapping areas of loss on each arm of chromosome 9, indicating that two distinct tumor suppressor loci are located on this chromosome. The deleted region on the p arm overlaps an area of 9p previously reported to be lost in a variety of neoplasms. Detailed loss of heterozygosity analysis of 9p in 112 primary bladder tumors using 12 microsatellite markers identified a minimal area of loss around the α-interferon locus at 9p21–22. Frequent homologous deletions of the α-interferon locus were then identified in these tumors by a novel, comparative, multiplex polymerase chain reaction assay and were subsequently confirmed by Southern analysis. Based on these deletions, a putative tumor suppressor gene locus involved in bladder tumorigenesis was localized to a 10 CM region (flanked by D9S162 and D9S171), previously implicated in the progression of many neoplasms. Application of the multiplex polymerase chain reaction-based assay will allow rapid identification of homologous deletions in many neoplasms and thus aid in mapping studies of critical suppressor genes.

Introduction
LOH of chromosome 9 is the most frequent genetic event yet described in bladder cancer and occurs in approximately 70% of bladder tumors tested (1, 2). Unlike other reported chromosome losses in bladder tumors (1, 3, 4), deletion of chromosome 9 occurs in all grades and stages of bladder carcinoma (2). Inactivation of a tumor suppressor gene on chromosome 9 is, therefore, a candidate initiating event in bladder carcinoma. Initial deletion mapping studies of bladder tumors reported a high percentage of monosomy and, less frequently, large partial deletions that spanned both arms (5–8). Although deletion of 9p21–22 was reported in a number of other tumor types (9–12), most deletion mapping studies of bladder tumors implicated a region of the q arm and proximal 9p excluding 9p21–22 (5–8). These observations raised the possibility that more than one tumor suppressor gene important in bladder carcinogenesis may map to chromosome 9. Recently, we provided evidence for two tumor suppressor genes on chromosome 9 derived from microsatellite analysis of LOH (13). Deletions of the p arm in some tumors and the q arm in others, clearly demonstrated two separate areas of loss on this chromosome.

In recent deletion mapping studies of chromosome 9 in bladder tumors, identification of the critical region has been hampered by the lack of selective or small deletions (5–8). Prior studies have shown the region of 9p21–22 surrounding the IFNα locus to be homologously deleted in leukemias (9), melanomas (10), gliomas (11), and lung tumor cell lines (12). This region, therefore, required careful analysis.

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3 LOH, loss of heterozygosity; IFNα, α-interferon; PCR, polymerase chain reaction.

Materials and Methods
Bladder Tumors and Constitutional DNAs. Primary tumor specimens were obtained by transurethral resection and frozen immediately. Peripheral blood from each patient was collected in EDTA as a normal control. Macroscopically pure tumor was dissected from the frozen biopsies and leukocytes were pelleted from blood samples before extraction and purification of DNA.

PCR Amplification. DNA from tumor and venous blood was analyzed for LOH by amplification of dinucleotide repeat-containing sequences using PCR and the conditions described (15). The markers used in this study are shown in Fig. 1. All primers were obtained from Research Genetics (Huntsville, AL). Primers were labeled with [γ-32P]ATP (DuPont NEN, MA) using T4 polynucleotide kinase (New England Biolabs, MA). Fifty ng of genomic DNA and 50 ng of each primer were subjected to 30 cycles of amplification. Annealing temperatures were between 54 and 58°C. For amplification of D9S165, Taq polymerase (Boehringer Mannheim, IN) was added only after samples were preheated to 95°C. Products were separated by electrophoresis in denaturing 7% polyacrylamide-urea-formamide gels followed by autoradiography (16). For informative cases, allelic loss was scored if the intensity of signal from one allele was significantly reduced in the tumor DNA when compared to the normal DNA.

Comparative Multiplex PCR. A primer set from a locus outside the area of suspected homologous deletion was selected as a control. Both sets of primers from the control locus and potentially deleted IFNα locus were included in the PCR reaction mix at a concentration of 2 ng/μl as described above. After amplification, an aliquot of amplified normal DNA was loaded alongside varying amounts of amplified tumor DNA. On the resulting autoradiograph, a tumor lane was chosen in which the signal of the retained allele(s) of the control locus was similar to that in the normal lane, and the intensity of the IFNα signal in the two lanes was compared. Homologous deletion was scored if the IFNα signal was less than 10% of the signal from the control allele(s).

Southern Analysis and DNA Probes. Restriction digestion of DNAs, blotting, labeling of the probe fragment, hybridization, and stripping of the filter were carried out as described previously (5). A 760-base pair EcoRI-XbaI fragment from phiIFNα (IFNα) and a 4.2-kilobase BamHI fragment from pEFD126.3 (D9S7) were used to probe Mspl digests. Polymorphisms and allele sizes were as described (17).

Results
Normal and tumor DNAs from 112 patients were screened for LOH on 9p with 12 microsatellite markers and on proximal 9q with six markers (Fig. 1). For this study, the q arm markers served to identify tumors with deletion of p only or separate deletions on p and q. Eleven
tumors showed deletion only on 9p, and eight tumors revealed discrete deletions of 9p and 9q separated by a region of retention. Of particular importance was a tumor (T46) which showed retention of heterozygosity at all informative loci on 9p and 9q, except at the IFNa locus at 9p21–22 where clear LOH was observed (Figure 2A). This tumor was informative for the two closest flanking markers to IFNa, D9S162, and D9S171, which retained heterozygosity. Another tumor (T3) showed a small deletion on 9p which included D9S171, IFNa and D9S162. In addition, 4 tumors had loss of p markers including, and distal to, D9S171 and IFNa, but retention of markers at 9p12–13 and proximal p21. Deletion mapping on 9p therefore delineated a critical region between D9S171 and D9S162 at 9p21-22 (Fig. 1).

Four tumors with large or complete deletions of 9p showed retention of heterozygosity at the IFNa locus (Figure 2B). This apparent retention of heterozygosity at a single locus within a large deletion suggested the possibility of homozygous deletion rather than two separate regions of loss on 9p. Multiplex PCR experiments with primers for both IFNa and D9S200 revealed that when the D9S200 allele(s) in the normal and tumor DNA lanes were of equal intensity, the signal for IFNa in the tumor lane was extremely weak. Diminution by >90% of the IFNa alleles in the same lane, where the intensity of the D9S200 control allele(s) was approximately equal, clearly demonstrated homozygous deletion (Figure 3A). Only gross overloading of the tumor DNA resulted in an IFNa signal of comparable intensity to that of the normal DNA lane. The signal from the deleted alleles in the tumor lane probably arises from normal cell contamination of the tumor biopsy. Initially, the IFNa signal appeared disproportionately strong because of the logarithmic amplification of the PCR.

To illustrate this further, a DNA template dilution assay which compared the effect of the amount of template DNA upon the strength of the autoradiograph signal was performed. Using as template, differing amounts of normal DNA from an individual homozygous at the IFNa locus, it can be seen from Fig. 3B that a 50-fold dilution of template DNA was necessary in order to observe a clear decrease (>50%) in signal intensity at the IFNa locus. Therefore, homozygous deletions will not be identified in tumors with significant amounts of contaminating normal DNA by a PCR-based assay.

Previous reports of homozygous deletion of 9p21–22 in tumor cell lines have demonstrated deletion by Southern analysis (9–12). To confirm the accuracy of our observations with PCR, Southern blots of MspI digests of normal and tumor DNA were probed with an IFNa complementary DNA to demonstrate homozygous deletion (Fig. 4). The filter was stripped and reprobed with a 9q marker, D9S7, as a control for loading differences between the normal and tumor DNA lanes. Absence of IFNa signal in the tumor lane on the Southern blot confirmed the presence of a homozygous deletion (Fig. 4). Once the validity of the PCR assay was established, careful multiplex PCR screening of the IFNa locus in other tumors with deletion of 9p revealed six other cases of homozygous deletion. In three of these cases, homozygous deletions extended beyond IFNa to include D9S171 in one case, D9S162 in another, and both D9S171 and D9S162 in the third case.

Discussion

Deletion of the IFNa locus and p arm loss, which presumably targets the same tumor suppressor gene, appear to be frequent events in bladder carcinoma. When found, homozygous deletions are thought to indicate close proximity of the target gene since both inactivation events are apparent. However, homozygous deletions have rarely been detected in human primary tumors. Indeed, only one case involving homozygous deletion of the RB gene has been previously reported in bladder carcinoma (4). Although uncommon, homozygous deletions in other cancers such as retinoblastoma (18), Wilms tumor (19), and colorectal tumors (20), have proven useful in cloning the target gene. Homozygous deletion of 9p21–22 has been described previously by Southern analysis (9–12) but predominantly in tumor cell lines where the amount of tumor DNA is not a limiting factor, and contamination with normal cells is not a problem. This study is the first to demonstrate the identification of homozygous deletions by PCR in primary tumors through the use of a simple, comparative multiplex assay.

Although the apparent retention of heterozygosity at a single locus within a large deleted region and a weaker signal from the tumor lane...
are suggestive of homozygous deletion, the possibility of retention and loading differences between lanes cannot be easily discounted. A multiplex PCR reaction controls for amplification and loading differences because the second primer set acts as an internal control. This technique clearly demonstrated homozygous deletion at the locus of interest in our tumors. It is worth noting that the incongruity of a small retention within a large deletion will only be noticed if flanking loci have been screened and are informative. Furthermore, since the majority of homozygous deletions we have detected do not extend beyond IFNa to flanking loci and this locus is only 50% informative, apparent retention within a large deletion cannot be used as a preliminary screen for homozygous deletions in many cases. In these non-informative cases, only comparative multiplex PCR or a more laborious Southern analysis can demonstrate homozygous deletion. Microsatellite dinucleotide analysis is now the method of choice for screening for LOH, and it is important to recognize the occurrence of homozygous deletions. In many neoplasms, DNA from the primary tumor is limited and insufficient for Southern analysis (i.e., where only archival paraffin blocks are available). Additionally, a small amount of normal cell contamination can produce a disproportionately strong residual signal. The DNA template dilution assay demonstrated that homozygous deletion could be misinterpreted as retention even in tumors with minimal (<10%) normal cell contamination. This comparative, multiplex PCR technique will be useful in identifying homozygous deletion at 9p21–22 and other regions in many types of primary tumors.

Deletions of the IFNa locus and p arm were found in all grades and stages of carcinoma including 3 Tis (grade 1) tumors. Inactivation of a tumor suppressor gene at 9p21–22 may, therefore, be an early event in bladder tumorigenesis. Homozygous deletions of IFNa were found in tumors with deletion of the p arm only, in those with distinct areas of loss on both arms, and also in tumors with apparent monosomy of chromosome 9. Without precise localization of the targeted region of 9q, it is impossible to know whether the putative tumor suppressor gene on the q arm is inactivated in these tumors. Six of the ten homozygous deletions were found in tumors with apparent complete loss of one homologue of chromosome 9. Since monosomy of chromosome 9 is significantly more common than partial deletion of chromosome 9 in bladder tumors, it is possible that the tumor suppressor gene at 9p21–22 is more frequently involved in bladder carcinoma than indicated by the number of homozygous deletions and p deletions so far detected. We are currently screening tumors with monosomy, and tumors with no apparent LOH on chromosome 9 for deletions around IFNa.

Because 7 of the 10 homozygous deletions of IFNa do not extend to the nearest flanking marker, the critical region must lie within a 10-cM region between D9S171 and D9S162 (21). Finer mapping of this region with closely spaced markers should further delineate the critical area. Deletion mapping is particularly important in bladder carcinoma because there is no defined or common familial form of this disease. Linkage analysis cannot, therefore, be used to identify the location of the initiating event(s). It now appears that loss of this critical region on 9p may be among the most common genetic changes in human cancer. The 10 homozygous deletions identified so far will prove valuable in precise mapping of this putative suppressor locus at 9p21–22.

**References**


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