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Advances in Brief

Homozygous and Hemizygous Deletions of 9p Centromeric to the Interferon Genes in Lung Cancer[1]

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Abstract

Cytogenetic analysis has indicated that deletion of chromosome 9p occurs in a significant number of non-small cell lung and mesothelioma tumors. Using paired oligonucleotide primers, we have undertaken an extensive analysis of 9p markers to determine homozygous and heterozygous loss of marker sequences. Homozygous loss of *D9S169* and *D9S171*, both of which map centromeric to the *IFN* gene cluster, were noted in three cell lines (27%) and hemizygous deletions of one or both of these loci was found in a further six cell lines (54%). These data suggest the presence of a potential tumor suppressor gene for lung cancer in proximity to *D9S169* and *D9S171* at 9p21.

Introduction

It is proposed that TSGs[4] play an important role in normal cells in the regulation of growth and differentiation and the inhibition of neoplastic development. Therefore the deletion and/or mutation of a TSG may contribute to the malignant transformation or progression of a cell. The role of TSGs in the formation of neoplasms is supported by the findings of nonrandom deletions of genetic material in a wide variety of tumor cells. We have detected loss of chromosome 9p as a nonrandom cytogenetic abnormality in 85% of cases of primary NSCLCs (1).[5] These data and similar findings of 9p deletions in other tumor types provide evidence for the hypothesis that one or more TSGs reside on the short arm of chromosome 9 and contribute to the malignant process.

Deletions of chromosome 9p have been observed at high frequency in various types of cancers. Both homozygous and heterozygous deletions which involved the loss of the *IFNA* or methylthioadenosine phosphorylase (*MTAP*) genes on chromosome 9p have been mapped in glioma and acute lymphoblastic leukemia tumor cells and lung cancer cell lines (2–6). Deletions of 9p including *IFNA* have also been reported in neoplastic hemopoietic cell lines (7). It has been noted, however, that loss of homozygosity in metastatic melanoma tumor and cell line DNAs was flanked by, rather than included, the *IFNA* locus (8) and that a locus frequently deleted in melanoma tumors, and presumably playing a critical role in predisposition to familial melanoma, is located near *IFNA* (9). Deletions in bladder carcinomas also were mapped centromeric to band 9p13, therefore excluding the interferon gene complex (10).

We have localized the loss of DNA from chromosome 9p in NSCLC and malignant mesothelioma (MM) cell lines in a previous study using Southern analysis. We concluded that the region between 9p13 and 9p22 was deleted in some cell lines and postulated that at least one TSG resides in this region (11). Reports have subsequently been published which describe 9p deletions both including the interferon genes and excluding them. Homozygous loss of the region 9p21–p22 including the *IFNA* locus has been reported in glioma cell lines (6) but the shortest region of overlap of 9p deletions of malignant mesothelioma cell lines was found to be centromeric to the interferon gene cluster (12).

In order to further define the region of loss of chromosome 9p we used PCR to screen NSCLC and MM cell lines for homozygous or heterozygous loss of DNA at 12 different loci positioned on 9p and for 1 locus positioned on 9q. Three of the 11 cell lines (1 NSCLC and 2 MM) showed homozygous loss of DNA at several of the 9p loci, and an additional 7 cell lines (4 NSCLC and 3 MM) had heterozygous loss of 9p as determined by scanning densitometry. From these results we have defined a common region of homozygous loss within chromosome band 9p21, thus further narrowing the minimum region and defining the location of the deletion. Our results also led to the clarification of the linear order of anonymous markers within chromosome 9p21, inasmuch as some ambiguities exist within this region according to recent publications from the International Workshop of Chromosome 9 (13).

Materials and Methods

Tumor Cell Lines. The NSCLC lines L111 and L112 were established in the Department of Cytogenetics, St. Vincent's Hospital, Melbourne, Australia; cell line A549 was obtained from the American Type Culture Collection (Rockville, MD); and the cell lines H520, H226, and H23 were derived at the National Cancer Institute (Bethesda, MD). All MM cell lines (LO68, NO36, ONE58, DeH128, and JU77) were supplied by Associate Professor Bruce Robinson from the Charles Gairdner Hospital, Perth, Western Australia. Lung cancer cell lines, rather than primary tumor tissue, were used in the study to eliminate contamination by normal cells.

Polymerase Chain Reaction. DNA was extracted from the cell lines using standard phenol/chloroform procedures, as described previously (14). As a positive control, genomic DNA was extracted from peripheral blood lymphocytes of a female colleague; the karyotype was 46,XX. Oligonucleotide primers were synthesized for 11 anonymous DNA markers localized to 9p (*D9S3, D9S52, D9S55, D9S104, D9S126, D9S161, D9S162, D9S163, D9S165, D9S169,* and *D9S171*) and one gene locus (*IFNA*) located between 9p13 and 9p22. Primers for the marker *D9S15*, assigned to 9q21, were also synthesized in order to use this locus as an internal control. The primer sequences for all of the chromosome 9 markers except *D9S3* were obtained by accessing the Human Genome Data Base; *D9S3* was sequenced by Robert Center.[6]

PCR reactions were performed using a Perkin-Elmer Cetus 480 thermal cycler in a total volume of 25 μl. PCR reactions were individually optimized for each pair of primers for annealing temperature, $MgCl_2$ concentration, and pH. The standard PCR reaction contained 10 ng of genomic DNA template, 25 pmol of each primer, 1 unit of Taq polymerase (Boehringer Mannheim, Mannheim, Germany), and a final concentration of 10 μM for each de-

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[4] The abbreviations used are: TSG, tumor suppressor gene; *IFNA*, interferon α gene; *IFNB*, interferon β gene; MM, malignant mesothelioma; NSCLC, non-small cell lung cancer; PCR, polymerase chain reaction.

[5] R. Lukeis, unpublished observations.

[6] R. Center, personal communication.

oxynucleotide triphosphate (Boehringer Mannheim). Standard PCR cycling was performed for most of the primer pairs as follows: denaturation at 94°C for 1 min; annealing at 50–65°C for 1 min; extension at 72°C for 1 min (for 30 cycles). The final extension was held at 72°C for a further 5 min. Amplification of *D9S161* and *D9S52* were achieved using a modification of the "touchdown" PCR technique (15). Six-tenths of each reaction was run on a 2% agarose gel, and the product was transferred to Hybond-N membrane (Amersham International, Buckinghamshire, Amersham, United Kingdom) and verified by Southern analysis for each marker, using purified, randomly labeled (Boehringer Mannheim) PCR product as probe. Filters were hybridized in a 50% formamide hybridization solution at 42°C for a minimum of 3 h and washed in 0.1× standard saline-citrate/0.1% sodium dodecyl sulfate (1× standard saline-citrate is 0.15 M NaCl-0.015 M trisodium citrate, pH 7.0) at temperatures between 42°C and 65°C, according to the stringency required. Filters were then exposed to X-ray film for approximately 8–16 h, to produce an exposure suitable for laser densitometry. Some polymorphism of PCR product size was observed with several of the markers due to variations of microsatellite DNA within the amplified regions.

PCR screening of all cell lines with each of the 13 loci was repeated twice. The resulting autoradiographs were then analyzed for allele dosage on a Molecular Dynamics Computing Densitometer 300A. The intensity of each PCR product was estimated by comparing the value of each cell line with the normal control, thus resulting in a ratio value for each band. The duplicate ratios were then averaged. PCR screening for the loci *IFNA*, *D9S126*, *D9S171*, *D9S169*, and *D9S161* were performed a total of four times with each cell line. The resulting four ratios for each of these loci were then averaged to enable us to determine heterozygous loss. A ratio value of 0.6–1.0 was considered a normal dosage and 0–0.4 as a hemizygous deletion.

Results

DNAs from each of 11 NSCLC and MM cell lines were subjected to PCR analysis for the presence of 12 different loci on chromosome 9p and 1 on 9q. Three of the cell lines showed a total absence of PCR product for several markers and hence were homozygously deleted for these sequences. The remaining eight cell lines produced PCR product for all primer pairs, indicating that all the markers tested were present on at least one allele of chromosome 9p in these cell lines (Figs. 1 and 2). Further analysis using laser densitometry then showed heterozygous loss of sequences in seven of these cell lines.

One NSCLC cell line (L111) and two MM cell lines (ONE58 and DeH128) showed homozygous loss of DNA for several 9p markers. NSCLC cell line L111 produced no PCR product for the markers *D9S3, D9S104, D9S161, D9S169,* and *D9S171*. MM cell line ONE58 produced no PCR product for *D9S126, D9S169,* and *D9S171*; and MM cell line DeH128 produced no PCR product for the markers *D9S3, D9S104, D9S126, D9S161, D9S169, D9S171,* and *IFNA* (Figs. 1 and 2). The common region of homozygous loss of DNA could be narrowed to within the band 9p21, between the markers *D9S126* and *D9S161* and encompassing *D9S171* and *D9S169* (represented by the *boxed area* of Fig. 2). Furthermore, heterozygous loss of one or both of the markers *D9S171* and *D9S169*, located in the common region of homozygous loss, was determined for three NSCLC cell lines (L112, H226, and H23) and three MM cell lines (LO68, NO36, and JU77) (Fig. 2). Taken together, these data indicate that the minimum region of deletion lies between *D9S126* and *D9S161*, although hemizygous deletions of chromosome 9 were identified between the *D9S15* and *D9S162* loci (Fig. 2).

Discussion

Lung cancer cell lines derived from NSCLC and MM tumors have been investigated for DNA deletions on chromosome 9p. We found homozygous loss of sequences in 3 of the 11 cell lines screened by PCR and heterozygous loss in a further 7 cell lines. A cell line was defined as having homozygous loss of DNA when the agarose gel and associated autoradiograph were totally lacking a detectable PCR product. Hemizygous deletion of genetic material was defined by laser densitometry, when the amount of PCR product produced in a cell line was less than four-tenths of the product produced by the positive control.

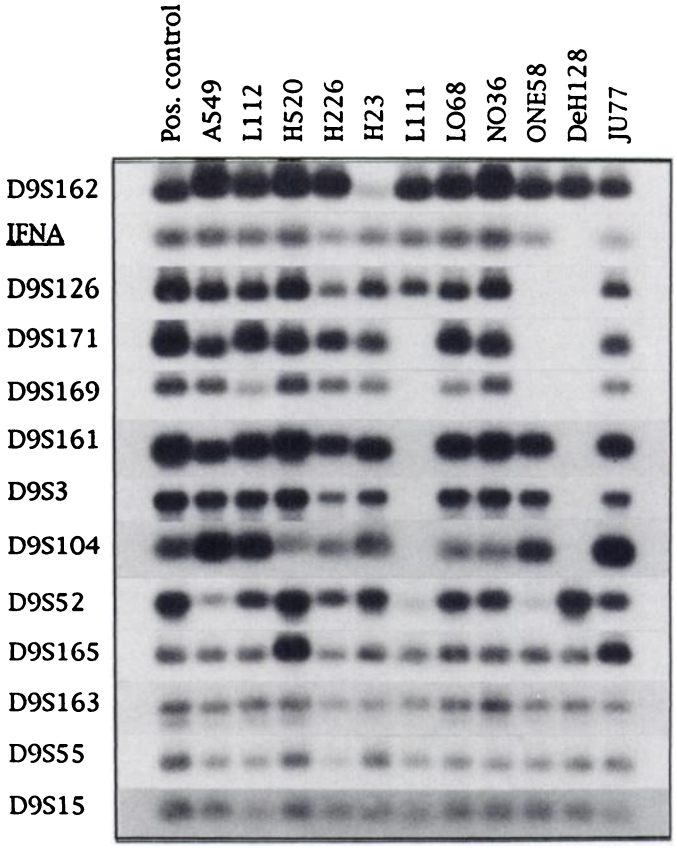

Fig. 1. PCR analysis of 9p markers in NSCLC and MM cell line DNA. Subsequent to PCR, products were resolved through a 2.0% agarose gel, transferred to nylon membranes, and hybridized with PCR-generated probes to each of the markers. *Pos. control,* normal control DNA isolated from peripheral blood leukocytes. 9p markers are arranged from centromeric (*D9S55*) to telomeric (*D9S162*); *D9S15* is a 9q marker.

In previous work we used Southern blotting analysis on the same series of NSCLC and MM cell lines to investigate four loci on chromosome 9p and found homozygous loss of DNA in two of the cell lines (L111 and DeH128) with heterozygous loss in a further five of nine cell lines (11). From this we estimated the deletions to lie between the bands 9p13 and 9p22. Our more recent analysis has extended the investigation of the same cell lines by using the more sensitive technique of PCR and a greater number of markers. This work has verified the homozygous losses of DNA found in the previous study and in addition has identified a region of homozygous loss in the MM cell line ONE58. Our more recent results have confirmed the hemizygous deletions previously found in three cell lines (H226, H23, and JU77) (11). Of the four cell lines that were described previously as having normal dosage (11), all except A549 now appear to contain smaller hemizygous deletions. The karyotypes of these cell lines (A549, L112, H520, and LO68) each showed two cytogenetically normal 9 chromosomes; hence we postulate that these contain much smaller deletions. The karyotypes of the remaining eight cell lines showed complex rearrangements involving the 9 chromosomes, with 9p deletions being cytogenetically observed in 5 of these.

The regions of loss found in this study either overlap the region of, or are mapped closely to, other deletions found covering the area from *D9S3* to *IFNA* at 9p21–22 (2–8, 10, 12). However, we have further narrowed the common region of deletion in NSCLC and MM to an

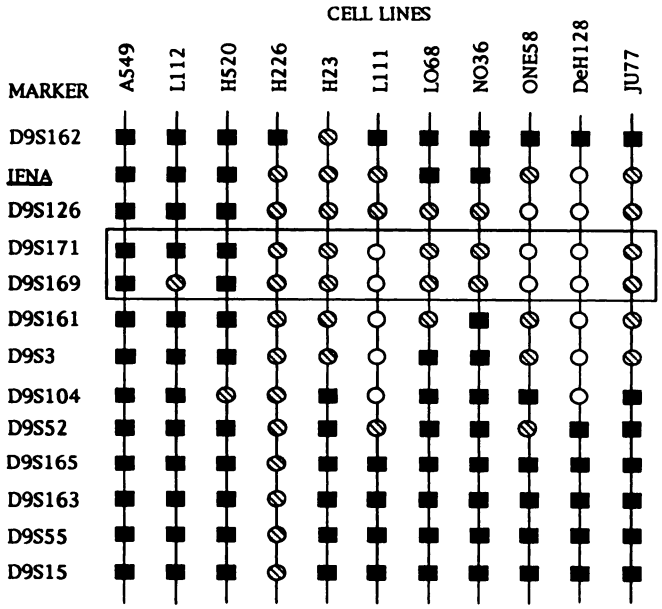

Fig. 2. Homozygous and hemizygous 9p deletions in NSCLC and MM cell lines. Markers: normal dosage (■, by densitometry >60% of normal control) and hemizygous (▨, by densitometry <40% of normal control) and homozygous (○) loss of marker. The common region of homozygous loss is *boxed*. 9p markers are arranged from centromeric (*D9S55*) to telomeric (*D9S162*); *D9S15* is a 9q marker.

area including only two loci, *D9S169* and *D9S171*, and contained within the chromosome band 9p21. It has been proposed that because the interferon gene cluster is frequently deleted from chromosome 9p it is therefore a candidate TSG. The *IFNA* cluster and *IFNB1* have been found to be deleted in many forms of neoplasms, and it has been claimed that deleted or inactivated *IFNA* genes, or a nonrelated gene which is closely linked to the interferon gene cluster, may play a role in carcinogenesis (2–7). However, our results from PCR screening of cell lines suggest that the *IFN* genes may not be a candidate TSG in NSCLC and mesotheliomas, since our defined narrowest common region of loss does not include the *IFNA* marker (the most centromeric of the *IFN* gene cluster). This suggests that the loss of DNA, and potentially a TSG, lies centromeric to the *IFNA* gene cluster in these cell lines. This assignment is in agreement with other deletions found in melanoma tumors (8), bladder cancer (10), and MM cell lines (12) which map centromeric to the *IFNA* gene cluster.

The linear order of loci established from our results confirms some and challenges other current mapping data. Based on the homozygous deletions of L111, ONE58, and DeH128 and the hemizygous deletions of L112, H226, H23, LO68, NO36, and JU77, we conclude that the markers should be mapped in the order: pter-*D9S126-D9S171-D9S169-D9S161-D9S3*-cen. This result differs from the map recently presented by Cheng *et al.* (12) who mapped *D9S171* telomeric to *D9S126*. Our results, like those of Cheng *et al.*, are based on the assumption that each region of loss consists of only one contiguous region of homozygous loss. However, if the order of loci was rearranged so that *D9S171* was telomeric to *D9S126*, this would imply that the NSCLC cell line L111 has a noncontiguous region of loss but would not alter the deletions mapped to the other cell lines. Thus, L111 could be interpreted as possessing two regions of homozygous deletion.

The map published from the most recent International Workshop on Human Chromosome 9 Mapping (13) showed an ambiguous region within band 9p21, which mapped *D9S169* centromeric to *D9S3* and could not position the marker *D9S161* relative to these. Our data define the proximal and distal limits of the deletions more precisely and clarify this area to be pter-*D9S169-D9S161-D9S3*-cen. Our results are in agreement with the order of other markers mapped at the International Workshop (13).

Clearly further study is required to localize and characterize one or more TSGs in the deleted region. Both NSCLC and MM are believed to have an etiology affected by environmental factors, such as cigarette smoke and asbestos. We hypothesize that such external factors cause a genetic event, such as a 9p deletion, in exposed cells, which results in the inactivation of a TSG and contributes to the neoplastic phenotype of the cell.

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