Distinct Deletions of Chromosome 9p Associated with Melanoma versus Glioma, Lung Cancer, and Leukemia

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Abstract

Deletions of DNA on chromosome 9p21–22 are frequently observed in cells derived from melanomas, gliomas, non-small cell lung cancers, and acute lymphoblastic leukemia. The minimal deletion shared by the latter three cancers extends from the interferon-α locus towards the centromere; its centromeric end is flanked by the gene encoding methylthioadenosine phosphorylase. We have determined that the telomeric end of the minimal homozygous deletion shared by two melanoma cell lines does not include the methylthioadenosine phosphorylase locus. Thus, a distinct region of DNA is lost in melanoma. The physical size of this region remains to be defined precisely, but it may extend over several million base pairs.

Introduction

Frequent, nonrandom chromosomal breaks and deletions that are characteristic of particular cancers may indicate the sites of tumor suppressor genes. Such a pattern has been observed on chromosome 9 in human malignant melanoma. Fountain et al. (1) have detected loss of heterozygosity for DNA markers within chromosome band 9p21 in more than 80% of melanoma tumor and cell line DNAs tested. The critical region is defined by the overlap between homozygous deletions in two melanoma cell lines, FF and AH, and is flanked by the IFN-α locus on the telomeric end and the D9S3 marker on the centromeric end (Fig. 1). Rearrangements on chromosome 9 are also characteristic of other cancers, including gliomas (2), acute lymphoblastic leukemia (3), and certain forms of lung cancer (4). The region of minimal overlap between deletions in a series of these malignancies has been defined by Olopade et al. (5–7) and by James et al. (2). The critical region here extends from the IFN-α locus towards the centromere with its centromeric end flanked by the gene encoding MTAP. The homozygous deletion in the melanoma cell line AH extends into the centromeric portion of the interferon gene cluster, raising the possibility that a single locus in the vicinity of MTAP might be implicated in all of these diverse cancers. However, we have determined that the telomeric end of the deletion found in the melanoma cell line FF does not include the MTAP locus. Thus, the region of overlap between the two melanoma deletions lies centromeric to MTAP and is distinct from that involved in glioma, lung cancer, and acute lymphoblastic leukemia. We have examined the chromosomes of these two melanoma cell lines and confirmed that a microscopically visible loss of chromosome 9p material has occurred in both of them. Pulsed-field gel analysis of DNA restriction fragments from these cell lines using probes that detect the IFN-α and D9S126 loci confirms the location of the telomeric 9p breakpoint in line AH between IFN-α and MTAP and suggests that the analogous breakpoint in line FF resides in a location centromeric to MTAP.

Materials and Methods

Cell Lines and Culture Conditions.

The origin of the human malignant melanoma cell lines FF (SK-Mel-133) and AH (SK-Mel-13) has been described (8). Cells were routinely maintained in Dulbecco’s modified Eagle’s medium plus 5% fetal calf serum without antibiotics. In some experiments, the culture medium was supplemented with 10 μM AZ and 20 μM MTA (both from Sigma Chemical Co.).

Immunoblot Analyses.

The presence of protein reactive with antisera directed against human MTAP was determined by an immunoblot method as described previously (9). Crude cell extracts (10–150 μg/lane) were separated by electrophoresis in a 12.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. Proteins were transferred by electroblotting to a nitrocellulose filter; nonspecific binding sites were blocked with 3% powdered milk in BBS (0.2 M sodium borate-0.15 M NaCl, pH 8.5), and the filter was incubated for 16 h at room temperature with anti-MTAP antisum diluted 1:500 in BBS containing 3% powdered milk. After the filter was washed with BBS, antibody was detected by binding for 1 h to 125I-labeled protein A (1 μCi/ml; ICN Radiochemicals, Irvine, CA), and the filter was exposed to Kodak XAR-5 film at −70°C. This procedure detects the M, 32,000 homotrimetric subunit of the MTAP protein.

Chromosome Studies.

Metaphase chromosome spreads were prepared and subjected to Giemsa-trypsin banding according to procedures described previously (10). Photomicroscopy was performed on a Nikon Optiphot microscope equipped with a 100X planapochromatic objective. Cytogenetic abnormalities are described according to the International System for Cytogenetic Nomenclature (11). In situ hybridization was carried out according to methods described in Ref. 12 using the chromosome 9-specific probe pBl-9 (kindly provided by Dr. Daniel Pinkel, University of California at San Francisco, San Francisco, CA). Following in situ hybridization, the biotin-labeled probe was visualized by binding to streptavidin conjugated with fluorescein isothiocyanate (Vector Laboratories). The chromosomes were counterstained with propidium iodide (Sigma) and observed under ultraviolet illumination through a Nikon B-2A filter (450–490 nm).

PFGE and DNA Hybridizations.

PFGE and Southern blotting were carried out as described (13). High molecular weight DNA (5 μg) was prepared in low melting temperature agarose plugs, digested with an excess (>30 units) of NotI or MspI restriction enzyme, and loaded into a contour-clamp homogeneous field electrophoresis gel consisting of 1.0% agarose. The gel was electrophoresed in TBE buffer (44.5 mM Tris-borate, 1 mM EDTA) at 10°C using a Pulsaphor unit (LKB) for 5 days at 90 V with a constant pulse time of 400 s. Afterward, the gel was exposed briefly to UV radiation (330 nm transmitted and 254 nm reflected for 2.5 min each), denatured in 0.5 M NaOH and 1.5 M NaCl, and neutralized in 1 M Tris-HCl and 1.5 M NaCl, pH 7.5. Transfer to a Zetabind membrane (AMF Cuno) was performed overnight in 10X SSC (1X = 150 mM NaCl-15 mM sodium citrate). Prehybridizations and hybridizations were carried out in a solution consisting of 50% formamide, 1 M NaCl, 25 mM Tris (pH 7.5), 1% Denhardt’s solution, 0.67% dextran sulfate, 0.1% sodium dodecyl sulfate, and 500 μg/ml sheared DNA from salmon testes. Probes were labeled by random primer extension, purified through a Sephadex G50-150
column, and added directly to the hybridization solution following 3 or more h of prehybridization at 42°C. Hybridizations were incubated overnight at 42°C and washed the following day under high stringency conditions (0.1X SSC, 0.1% sodium dodecyl sulfate at 65°C). Blots were exposed to Kodak XAR-5 film for 1-5 days at ~70°C. For subsequent hybridizations, blots were stripped in hybridization solution at 65°C for 1 h, followed by a 30-min wash in 0.1X SSC and 0.1% sodium dodecyl sulfate at 65°C.

The probe p72-0.9 was used to detect the D9S126 locus (13), and a 320-base pair polymerase chain reaction product was used to detect the IFN-α gene cluster. The primers used to amplify this product were (14): IFNA 203: 5’-GGC TGC TC-3’ and IFNA 523: 5’-GGA TCT CAT GAT Trc TGC TC-3’.

These primers were chosen from highly homologous regions found between the IFN-α genes in an effort to maximize the number of IFN-α genes detected by the probe. On conventional Southern blots, this probe recognizes essentially the same pattern as that depicted in Fountain et al. (1). Where, alternatively, a cloned IFN-α fragment was employed as a probe (data not shown).

Results

Fig. 1 shows the order of marker loci in chromosome band 9p21-22. The order of the markers D9S33, IFNB, IFNA, D9S126, D9S3, and D9S19 has been established by a combination of multipoint linkage analysis, fluorescence in situ hybridization, and PFGE (13). The IFNB gene and the IFNA gene cluster, which includes about 20 genes and pseudogenes, reside on a single, 1400-kilobase NotI restriction fragment (6). The physical distances between the other markers remain undetermined. The position of the MTAP locus in relation to the IFN genes has been established through an analysis of deletions in a series of leukemia, glioma, and lung cancer cell lines and tumors (6, 7). Cytogenetic analysis of del(9p) chromosomes or unbalanced translocations involving 9p in cell lines with homozygous partial deletions of IFN DNA sequences localized the IFNB gene telomeric to the IFNA gene cluster, and cell lines with deletion breakpoints within the IFNA gene cluster established the order of some of the genes within the cluster. Many of the cell lines with deletions of the centromeric portion of the IFNA gene cluster also lost MTAP enzyme activity as determined by a radiochemical assay (6, 7). The cell lines with the smallest deletions indicated that the minimal deleted region shared by all these tumors lies between the centromeric end of the IFNA gene cluster and the MTAP gene.

Location of the MTAP Locus Relative to Homozygous Deletions in Melanoma Cell Lines. The homozygous deletion in melanoma cell line AH extends as far as the centromeric portion of the interferon gene complex. This cell line retains only one allele of the IFNA locus, and this allele has suffered a rearrangement detectable by Southern hybridization (1). Since the D9S126 locus, centromeric to MTAP, is also homozygously deleted in cell line AH, we predicted that the MTAP gene would be lost as well. If so, this deletion would overlap the region identified in lung cancer, glioma, and acute lymphoblastic leukemia (Refs. 6 and 7; see Fig. 1). However, the telomeric extent of the homozygous deletion in cell line FF, which includes the D9S126 and D9S3 loci, has yet to be determined.

No DNA probes corresponding to the MTAP gene were available for hybridization studies. We therefore examined the two melanoma cell lines for evidence of the MTAP gene product. A preliminary experiment tested the ability of the cells to survive in culture medium containing MTA and AZ. AZ is an inhibitor of de novo purine biosynthesis. The MTAP enzyme permits a cell to use MTA as a source of purine nucleotides for DNA synthesis via nucleotide salvage pathways, and thus it confers the ability to survive under these selective conditions (15). The two cell lines exhibited markedly different behaviors in this selective medium. AH cells failed to grow in MTA+AZ at any cell density tested but grew well under nonselective conditions. FF cells grew poorly at low cell densities whether or not the inhibitor was present, but at higher cell densities they grew vigorously even in the presence of MTA+AZ. This indicated that FF cells might retain the MTAP gene.

To confirm the presence of the MTAP gene product, we performed immunoblots of melanoma cell extracts with an antibody directed against human MTAP (Fig. 2). No immunoreactive protein was found in extracts of AH cells, consistent with their inability to survive in MTA+AZ. Protein reactive with the anti-MTAP antibody was, however, clearly evident in the extract of FF cells. The ability of FF cells to survive in MTA+AZ indicates that this immunoreactive material is functional MTAP.

Chromosome 9 Abnormalities in Melanoma Cell Lines AH and FF. Both of these cell lines have sustained loss of heterozygosity and homozygous deletions of polymorphic DNA markers within chromosome band 9p21-22 (1). We examined the chromosomes of these two cell lines using G-banding and confirmed the identity of marker chromosomes derived from chromosome 9 by in situ hybridization with a probe specific for human chromosome 9 (Fig. 3). As suspected, neither cell line contains an intact copy of chromosome 9. The melanoma cell line AH contains two marker chromosomes derived from chromosome 9; one resembles a copy of chromosome 9 with a terminal deletion of the short arm (Fig. 3A, marker chromosome M8), and the other is an apparent iso9q chromosome in which a portion of one arm has been duplicated (Fig. 3A, marker chromosome M1). The homozygous deletion in this cell line includes the D9S126 locus, and there
Fig. 3. Deletions of chromosome 9 in melanoma cell lines AH and FF. (A) Giemsa banded karyotype of AH. Arrows, two marker chromosomes derived from chromosome 9. One representative metaphase spread is depicted. In twenty-five metaphase spreads examined, the total number of chromosomes varied from 76 to 102. The chromosome abnormalities we observed in AH melanoma cells can be summarized: XX[1]; XXX[7]; XXXX[14]; XXXXX[3]; +[17]; +1x2[17]; +1x3[1]; +2[18]; +2x2[3]; +3[9]; +3x2[15]; +36[1]; +4[22]; +4x2[2]; +58[1]; +5x2[17]; -6[3]; +7[6]; +7x2[13]; +7x4[1]; +7x5[1]; +8[20]; +8x2[2]; -9x2[25]; -10[1]; +10[3]; +11x2[8]; +11x3[1]; -12[4]; +12[2]; +12x2[1]; -13[2]; -13x2[23]; -14[1]; -14x2[24]; +15[11]; +15x2[11]; +15x3[11]; +15x4[11]; +16[8]; +16x2[16]; +16x3[1]; -17[1]; -17x6[1]; +17x7[1]; +18[19]; +18x2[2]; -19[1]; +19[15]; +19x2[1]; +19x3[1]; +20x4[11]; +20x5[10]; +20x6[4]; +21[3]; +21x2[4]; +21x3[8]; +21x4[4]; +21x5[3]; -22[2]; +22[21]; +22x2[1]; +22x3[1]; +M1[24]; +M1x2[7]; +M2[20]; +M3x2[1]; +M4[1]; +M4x2[11]; +M4x3[11]; +M4x4[2]; +M5[22]; +M5x2[1]; +M6[25]; +M7[24]; +M7x2[1]; +M8[23]; +M8x2[1]; +M9[6]; +M9x2[1]; +M10[3]; +M10x2[21]; +M10x3[1]; +M11[19]; +M11x2[2]; +M12[21]; +M12x2[1]. (A plus sign denotes the presence of extra copies of a chromosome; for example, +19x2 signifies two copies of chromosome 19 in addition to the normal diploid complement. A minus sign denotes the loss of a chromosome. The numbers in square brackets indicate the number of spreads in which the abnormality was observed. M1, M2, etc. refer to the marker chromosomes depicted in the photograph. The abnormalities involving chromosome 9 and the marker chromosomes derived from it have been underlined.) (B) Metaphase chromosomes of AH melanoma cells hybridized in situ to chromosome 9-specific probe pBL-9. The biotin-labeled probe was visualized with avidin conjugated to fluorescein isothiocyanate (bright fluorescence), and the chromosomes were counterstained with propidium iodide. Material derived from chromosome 9 is evident on marker chromosomes M1 and M8. (C) Giemsa banded karyotype of FF. Arrows, copies of chromosome 9 with deletions of a portion of the short arm. This karyotype is a composite; it includes chromosomes from one representative metaphase spread plus marker chromosomes M1, M2, M3, and M19 from additional spreads. The number of chromosomes per cell varies in twenty-five metaphase spreads varied from 71 to 83. The summary of karyotypic abnormalities we observed in FF melanoma cells is as follows: XY[20]; XYY[1]; XXY[1]; X(Y;2]; +1(17); -2[4]; -2x2[1]; +3[25]; +4[22]; +5[15]; +6[22]; +7[20]; +7x2[4]; +8[21]; +8x2[1]; -9x2[25]; +10[20]; +10x2[1]; +11[25]; +11x2[1]; +12[4]; +12x2[20]; +13[9]; +13x2[12]; +14[22]; +19[2]; +15x2[2]; +16[6]; -16[2]; +17[1]; -17[6]; +18[19]; -18[1]; +19[6]; +19x2[11]; +19x3[5]; +20[14]; +20x2[2]; +21[11]; +21x2[9]; +21x3[2]; +21x4[1]; -21[1]; -22[10]; +22x1[1]; -22x7[1]; -22x2[1]; +M1[22]; +M1x2[1]; +M2[20]; +M2x2[1]; +M3[4]; +M4[19]; +M4x2[1]; +M5[21]; +M6[24]; +M7[24]; +M8[24]; +M8x2[1]; +M9[6]; +M10[18]; +M10x2[2]; +M11[5]; +M12[18]; +M13[22]; +M14[19]; +M15[16]; +M15x2[1]; +M16[21]; +M17[14]; +M18[5]; +M19[2]. (D) Metaphase chromosomes of FF melanoma cells hybridized in situ to pBL-9. Material derived from chromosome 9 is evident on at least two chromosomes (arrows) in addition to marker chromosomes M7 and M8.

is loss of heterozygosity at loci both centromeric (D9S3) and telomeric (IFNA, IFNB1, and D9S33) to D9S516. However, since at least one allele at both of these flanking loci is retained, the appearance of the chromosomal deletions cannot be accounted for by a simple deletion of both 9p termini. Cell line FF has a homozygous deletion that encompasses two loci, D9S126 and D9S326. It contains two nearly identical marker chromosomes that resemble terminal deletions of 9p (Fig. 3C). This cell line also retains alleles at loci both centromeric and telomeric to the homozygous deletion, but in contrast to AH, in situ hybridization revealed a complex arrangement of material derived from chromosome 9 that was translocated onto other chromosomes (Fig. 3D).

Twenty-five metaphase chromosome spreads from each cell line were karyotyped. Both of these cell lines are near-tetraploid with a highly variable number (70–100) of chromosomes. No intact copies...
NotI breakpoints in AH and FF, these melanoma DNAs were digested with MluI and NotI restriction endonucleases, separated on a PFGE, and hybridized to probes for IFN-α and D9S126 (Fig. 4). All of the genes corresponding to the IFN-α locus reside on a single 1.4-megabase NotI restriction fragment (5). A fragment of 1.4 megabases is seen in FF (Mel), but in AH (Mel) a faster-migrating band of 1.2–1.3 megabases is evident. The IFN-α probe hybridized to this blot detects IFN-α genes located up to and within approximately 100 kilobases from the centromeric end of the 1.4-megabase NotI fragment. Thus the breakpoint within the IFN-α locus in AH (Mel) probably occurred within this region. IFN-α genes located up to and within approximately 100 kilobases from the centromeric end of the 1.4-megabase NotI fragment. Thus the breakpoint within the IFN-α locus in AH (Mel) probably occurred within this region.

When the same PFGE blot was hybridized to a probe corresponding to the D9S126 locus, only one very faint MluI fragment was observed within the zone of resolution. Since D9S126 is homozygosly deleted in both FF (Mel) and AH (Mel), no signal was expected in either one of these two lanes. However, D9S126 fragments are present in DNA from the lymphocytes of patient FF (1), and a faint MluI band is seen in the FF(B) lane in Fig. 4. In general, however, the NotI and MluI restriction fragments corresponding to D9S126 are larger than 1.6 megabases and, therefore, were not resolved on this blot.

Discussion

There are now several lines of evidence for the involvement of a chromosome 9 tumor suppressor in human malignant melanoma. Cytogenetic studies have revealed rearrangements of chromosome 9 in both melanomas and precursor lesions, suggesting that damage to this chromosome may be an early event in melanocytic tumor progression (16). This suggestion was also supported by early molecular studies performed on multiple metastatic cell lines derived from a melanoma patient (17). In recent studies, the targeted region has been more precisely defined on 9p21–22. Attention has focused on the DNA marker D9S126 since loss of heterozygosity has most frequently been detected by this marker in the vast majority of melanoma cell lines and tumors examined (1). In addition, a melanoma patient has been described who harbors a constitutional deletion of a portion of chromosome 9p21; she has developed eight primary melanomas, atypical moles, and a plexiform neurofibroma (18). This patient is hemizygous for chromosome 9p21 DNA loci between D9S33 and D9S19, markers which lie just telomeric and just centromeric to the region of homozygous deletion detected in the tumor cell lines AH and FF. Finally, a hereditary predisposition to melanoma has been linked to chromosome 9p21 markers in several melanoma pedigrees (19), although there is also evidence for genetic heterogeneity in this disease (20).

The discovery of deletions at the interferon locus in a high proportion of acute lymphoblastic leukemias (3), and later in gliomas (2) and non-small cell lung cancers (4), led to the proposal that loss of inter-feron gene function might play a role in the development of these cancers. However, careful mapping of the deletions shared by a large number of tumor samples has placed the critical region in an area centromeric to the interferon gene cluster (5–7). In a substantial number of tumors with smaller deletions, the MTAP locus is also retained, and at present this locus marks the centromeric boundary of the region of overlap. It now appears that the region frequently deleted in these tumors is distinct from that in melanoma. This may indicate that two different tumor suppressor genes are targeted in these cancers, although the possibility that a single very large locus extends through the MTAP gene has not been excluded.

Current estimates of the size of the homozygous deletion shared by the FF and AH melanomas range from 2–5 megabases. The present PFGE study demonstrates only that the IFNA and D9S126 loci reside on separate, large restriction fragments. The karyotypes of the two cell lines raise the possibility that the deletions could be quite large since both have large-scale deletions and clearly visible cytogenetic rearrangements of telomeric chromosome 9p. However, these rearrange-ments are certainly more complex than simple terminal deletions since some 9p21–22 DNA loci are retained in both cell lines. Further physical mapping surrounding the IFNA, MTAP, and D9S126 loci will be required to determine the precise extent of the critical region for each cancer.

References


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