A Malignant Melanoma Tumor Suppressor on Human Chromosome 11

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ABSTRACT

Considerable molecular genetic and cytogenetic evidence indicates that chromosome 11 is a target for chromosome breakage, rearrangement, and loss during the development of human malignant melanomas. Abnormalities of the long arm of chromosome 11 are also evident in a wide variety of other human solid tumors, including carcinomas of the breast, ovary, cervix, and lung. In melanomas, these abnormalities tend to cluster in the lower half of the long arm of chromosome 11, indicating the possible presence of a melanoma tumor suppressor gene in this region. We tested this possibility by using microcell-mediated chromosome transfer to introduce normal copies of human chromosome 11 into two human malignant melanoma cell lines. In one cell line, MeJuSo, the presence of an additional copy of chromosome 11 severely reduced the ability of the cells to grow in culture. In a second cell line, UACC 903, there was a moderate reduction in cell growth in vitro, and the ability of the hybrid cells to form tumors in animals was suppressed. Suppression of tumorigenicity was even more strongly pronounced in a microcell hybrid that received an isochromosome 11q derived from the donor copy of chromosome 11. The formation of tumors was accompanied by a reduction in the copy number of chromosome 11. This provides functional evidence that a melanoma tumor suppressor resides on the long arm of chromosome 11. Thus, a third distinct locus, in addition to those previously defined on chromosomes 6 and 9, appears to play a role in the development of human malignant melanoma.

INTRODUCTION

Malignant melanoma is the one form of skin cancer associated with a significant risk of mortality from metastatic disease, and its incidence is rising faster than that of any other cancer in the United States (1). Early primary melanomas pose a very low risk of recurrence if they are excised while the pattern of tumor growth remains confined to radial expansion within the uppermost layers of the skin (2). Primary melanomas which have progressed to a pattern of vertical growth, invading the deeper layers of the dermis, are associated with a much higher risk of metastasis. The transition from the radial to the vertical growth phase marks a significant change in many of the biological properties of a melanoma tumor (3). At this stage, tumor cells grow indefinitely in culture, become independent of exogenous growth stimuli required by normal melanocytes, and acquire the ability to form tumors in animals. The cells also clearly manifest genetic instability, and many of the chromosome abnormalities characteristic of melanomas make their first appearance at this time. Some of these genetic alterations must underlie the changes in cell growth properties that emerge in the vertical growth phase.

Abnormalities of chromosomes 9 and 10 may occur during very early stages of melanoma tumor formation, since they have been reported in primary melanomas and occasionally in premalignant nevi (4). Mutations in a candidate tumor suppressor gene, the cyclin-dependent kinase inhibitor 2 gene, have recently been identified in a large proportion of families with familial melanoma linked to chromosome 9 (5). The structural chromosome abnormalities found most frequently in regional metastases are those involving chromosomes 1, 6, 7, and 11 (6, 7). Chromosome 7 usually undergoes duplication. All of the rest typically suffer breaks and deletions, which suggests that tumor suppressor genes on these chromosomes may be the targets of inactivating mutations. Chromosome transfer studies have provided functional evidence for a melanoma tumor suppressor locus on chromosome 6 (8).

The cytogenetic evidence is consistent with the presence of a melanoma tumor suppressor on chromosome 11 as well. A study of clonal chromosomal structural abnormalities in a series of 158 melanomas detected a significant clustering of chromosome breakpoints on chromosome 11 at 11q23-ter (7). This result is supported by earlier cytogenetic reports (9) and by molecular studies of LOH for polymorphic markers in melanoma tumor DNAs. Two independent surveys of melanoma LOH have revealed a relatively high frequency of loss at the APOC3 and D11S29 loci located in chromosome band 11q23 (9-11). Finally, there is evidence for an association between the presence of chromosome 11 abnormalities and poor clinical outcome in melanoma (12).

In the present study, we investigated whether human chromosome 11 could reduce the tumorigenicity of two human malignant melanoma cell lines, UACC 903 and MeJuSo, by performing microcell-mediated chromosome transfer of a normal human chromosome 11. The introduction of an intact copy of chromosome 11 caused a sharp reduction in either cell growth or tumorigenicity in these two melanoma cell lines. This effect was enhanced in cells that received an isochromosome 11q. Thus, we provide functional evidence that human chromosome 11 carries a tumor suppressor locus involved in malignant melanoma. Moreover, our observations indicate that the suppressor is located on the long arm of the chromosome and appears to function in a dose-dependent manner.

MATERIALS AND METHODS

Cell Culture. The human malignant melanoma cell lines used as the recipients for chromosome transfer were UACC 903 (8), obtained from Dr. Jeffrey Trent (National Center for Human Genome Research, NIH, Bethesda, MD), and MeJuSo (13), obtained from Dr. Judith Johnson (Institute for Immunology, Munich, Germany). Both of these cell lines were originally derived from metastatic human melanomas. The chromosome 11 donor cell line HDm-18 is a hybrid mouse 3T6 cell line containing human chromosome 11 tagged with an integrated neo gene (14). All cell lines were maintained in DME (Mediatech, Inc., Washington, DC) supplemented with 5% fetal bovine serum (Gemini Bioproducts, Calabasas, CA). Melanoma cells and microcell hybrid cell lines were monitored for Mycoplasma contamination by solution hybridization using a kit from Gen-Probe (San Diego, CA).

Chromosome Transfer. Microcell-mediated chromosome transfer was performed as described (14). A 48-h exposure to 0.02 µg/ml Colcemid was used to induce micronucleation in the chromosome 11 donor cell line. After fusion with microcells, recipient melanoma cells were subcultured into selective medium containing 0.5 mg/ml G418 (Geneticin; Life Technologies, Inc., Gaithersburg, MD) and incubated for 4 to 6 weeks until colonies of drug-resistant cells were visible.

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2 The abbreviations used are: LOH, loss of heterozygosity; FISH, fluorescence in situ hybridization.

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resistant cells appeared. Colonies were isolated by trypsinization in cloning rings. Hybrid cell populations were expanded and maintained under selective conditions in medium containing 0.5 mg/ml G418.

Chromosome Analysis. Preparation of G-banded karyotypes was carried out according to the procedures described elsewhere (15). A minimum of 20 metaphase spreads were photographed under a Zeiss Planapochromat X100 objective and karyotyped for each cell line. FISH was done as described previously (16). The probe for mouse chromosomes consisted of total mouse DNA isolated from mouse A9 cells (17) and was biotinylated via nick translation using a Bio-Nick kit (Life Technologies, Inc.) according to the manufacturer’s instructions. The chromosome 11 centromere-specific probe D11Z1 was purchased from Oncor, Inc. (Gaithersburg, MD), and chromosome 11 painting probe was purchased from Vector Laboratories (Burlingame, CA).

Cell Growth and Tumorigenicity Assays. To measure cell growth rates in culture, 10³ cells were plated into 60-mm tissue culture dishes; on 4 successive days, the cells in triplicate dishes were counted on a hemacytometer. The population doubling time was calculated from the exponential portion of the growth curve. Assays for anchorage-independent growth were carried out by plating cells in 0.3% noble agar as described previously (18). For tumor assays in athymic mice, 5 x 10⁶ cells suspended in 0.2 ml of DMEM with 5% fetal bovine serum were injected s.c. into the flanks of 4-6-week-old BALB/c-nu/nu mice (18). Each mouse received an injection in both the right and left flanks. Animals were examined at approximately 7-day intervals for 1 to 2 months. The dimensions of developing tumors were measured with a millimeter ruler, and the sizes of the tumors were estimated in units of mm³. In some cases, after the animals were euthanized, tumor cells were harvested aseptically for culture in vitro.

RESULTS

Transfer of Chromosome 11 Reduces Cell Growth. To determine whether human chromosome 11 carries a melanoma tumor suppressor gene, we transferred intact normal copies of chromosome 11 into two human malignant melanoma cell lines by microcell-mediated chromosome transfer. The chromosome 11 donor cell line used in these transfer experiments was HDm-18, a hybrid mouse 3T6 cell line containing human chromosome 11 tagged with a neo selectable marker. The selectable marker is integrated into chromosome 11 in approximately 35% of the cell population (data not shown).

The human malignant melanoma cell lines UACC 903 and MelJuSo have been characterized cytogenetically (8, 19). Both are near-diploid; the populations of UACC 903 and MelJuSo used in this study had modal chromosome numbers of 47 and 48, respectively. The karyotype of the UACC 903 cells was 47, X, -1, -2, -4, +7, -8, -9, -11(2), -14(2), -15, -17, -19, -20 plus marker chromosomes M1 through M12. The selectable marker is integrated into chromosome 11 at band 1q16 (14). This chromosome donor cell line also contains an iso(chromosome 11q1 derived from human chromosome 11 in approximately 35% of the cell population (data not shown).

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Detailed analysis of the chromosome 11 content in each cell line by G-banding revealed that MelJuSo retained two apparently normal copies of chromosome 11. In UACC 903, on the other hand, both copies of chromosome 11 had undergone alterations of the q arm in the 11q22-q23 region, which are depicted in Fig. 1. Marker chromosome M2 was derived from a translocation between chromosome 11 and chromosome 1 (der(1;11)(q21;q22)), whereas marker chromosome M13 involved a translocation with an unidentified chromosome [der(11;?)q23;?]). Thus, marker M2 had lost a portion of chromosome 11 from 11q22 to the q-terminal region, whereas marker M13 had lost a portion of chromosome 11 from 11q23 to qter.

The G-banding analysis was confirmed using FISH with a chromosome 11-specific chromosome painting probe (Fig. 2). Hybridization to MelJuSo chromosomes revealed two intact painted chromo-
had been transferred and to verify that the chromosome content of the hybrid cells, with the exception of the transferred chromosome, was the same as that of the parental melanoma cell line. Finally, for each microcell hybrid, 200 cells were scored following FISH with a chromosome 11 centromere-specific probe, D11Z1. This procedure enabled us to reliably determine the proportion of the hybrid cell population which retained the additional copy of chromosome 11.

Six microcell transfers of chromosome 11 into MeLiSo cells produced a total of 83 G418-resistant colonies. Only 14 of these colonies could be expanded to provide sufficient cells for analysis using FISH, and all but three of these were eliminated because of the presence of donor-derived mouse chromosomes. These three hybrids had a dramatically altered morphology (Fig. 3) and grew extremely slowly in vitro. Hybrid MeL(11n)34 was examined with FISH using the chromosome 11 centromere-specific probe, which indicated that the transferred chromosome was present in 76% of the cell population. This hybrid and the others died out before any further analysis could be performed.

A single transfer of chromosome 11 into UACC 903 produced four G418-resistant hybrids, of which three were free of mouse chromosomes. The 903(11n)1 and 903(11n)2 hybrids had a modal chromosome number of 48, and their karyotypes were identical to those of UACC 903 with the addition of an intact copy of chromosome 11. Hybrid 903(11n)4 also had a modal chromosome number of 48, but in this case the additional chromosome was an isochromosome 11q. Recovery of microcell hybrids with iso11q chromosomes following transfer from the HDM-18 donor cell line has been reported previously (20). The genetic analysis of UACC 903 and the 903(11n) microcell hybrids is summarized in Table 1. Representative G-banded chromosomes are shown in Fig. 1, and Fig. 2 depicts the results of chromosome painting with a chromosome 11-specific probe. FISH with the chromosome 11 centromere-specific probe was used to monitor the hybrid cell populations to determine whether they retained or lost the additional copy of chromosome 11 following passage in vitro under selective conditions (data not shown). All three UACC 903 microcell hybrids stably maintained the transferred chromosome.

The growth rate of UACC 903(11n) microcell hybrids in vitro was distinctly slower than that of the parental melanoma cells. The typical doubling time for exponentially growing UACC 903 cells was 28 h, whereas the doubling times for hybrids 903(11n)1, 903(11n)2, and 903(11n)4 were 44 h, 40 h, and 44 h, respectively. Thus, in two independent malignant melanoma cell lines we observed severe or moderate cell growth suppression following introduction of human chromosome 11.

Suppression of Tumorigenicity in 903(11n) Microcell Hybrids. All three 903(11n) microcell hybrids exhibited a clear reduction in the ability to form tumors in athymic mice. Introduction of the neo gene by itself has no effect on the tumorigenicity of UACC 903 cells (8). The time course of tumor formation by UACC 903 and microcell hybrid cells is shown in Fig. 4. The parental human melanoma cells formed tumors rapidly. The latent period for tumor formation was extended by approximately 50% in hybrids 903(11n)1 and 903(11n)2, which were similar to each other. Tumor formation by hybrid 903(11n)4, which contained a transferred isochromosome 11q, was even more suppressed. This hybrid cell line required twice as long to form tumors as the parental UACC 903 cells did. These results are summarized in Table 2.

Microcell hybrid 903(11n)4 was distinguished from hybrids 903(11n)1 and 903(11n)2 by two additional characteristics. Although the two microcell hybrids that received an intact copy of chromosome 11 differed very little in appearance from the parental UACC 903 melanoma cells, hybrid 903(11n)4 exhibited a noticeable shift in morphology. The cells of this hybrid were distinctly larger and flatter than those of the other two hybrids or the parental melanoma cell line. Hybrid 903(11n)4 also exhibited a marked reduction (>100-fold) in the ability to form anchorage-independent colonies when plated in soft agar. This form of growth suppression was not evident in hybrids 903(11n)1 and 903(11n)2 (Table 2).

Cells derived from tumors formed by 903(11n) microcell hybrids were returned to in vitro culture under nonselective conditions and analyzed for their chromosome 11 content. First, FISH with the chromosome 11 centromere-specific probe was used to measure the percentage of the tumor cell population that retained an additional copy of chromosome 11. All of the tumors exhibited a reduction of at least 50% in the proportion of cells with three chromosome 11 centromeres. Then G-banded chromosomes were examined to determine whether the reduction was due to loss of the transferred copy of chromosome 11 or one of the parental marker chromosomes.

The results are summarized in Table 3. In two tumors derived from hybrid 903(11n)1, about 50% of the cell population had lost the transferred intact copy of chromosome 11. In two tumors arising from hybrid 903(11n)2, only 10–20% of the cells retained the additional chromosome, but in both of these tumors marker chromosome M13 was lost and the transferred intact chromosome 11 was retained. Two tumors formed by hybrid 903(11n)4 exhibited loss of the transferred isochromosome 11q in more than 90% of the cell population. In a
Fig. 4. Suppression of tumor formation in 903(11n) microcell hybrids. Growth of UACC 903 melanoma cells and 903(11n) microcell hybrids following injection into athymic mice: UACC 903 (●), 903(11n)1 (▲), 903(11n)2 (▲), and 903(11n)4 (▲). Values are averages of 11–20 injection sites in 5–10 mice/cell line; bars, SE.
includes an intact 11q arm. In this hybrid, which received two extra copies of 11q rather than one, the latent period for tumor formation was twice as long as in the other two 903(11n) microcell hybrids. This hybrid also displayed more pronounced effects on cell shape and on capacity for anchorage-independent growth.

The pattern of chromosome loss in tumors derived from the other two hybrids is also consistent with a dosage effect (21). In all cases, tumor growth was associated with a net loss of chromosome 11 material. In one case the transferred intact copy of the chromosome was lost, whereas in the other case the marker chromosome with the larger portion of the 11q arm was eliminated. Although no definitive statement can be made, it is tempting to speculate that the marker chromosome with the smaller portion of chromosome 11 has suffered a deletion of the region containing the tumor suppressor locus, so that the UACC 903 cell line retains only a single copy of it. A dosage effect might explain the relatively severe growth suppression that occurred following transfer of chromosome 11 into MelJuSo cells. These cells have two intact copies of the chromosome and may remain diploid for the suppressor locus. However, this remains speculation since the MelJuSo cell line has not been investigated for submicroscopic alterations on chromosome 11. It is also possible that this cell line allows for higher expression of the suppressor locus function on the introduced chromosome than does the UACC 903 cell line.

It may also be significant that the region of chromosome 11q missing from the marker chromosome retained in the tumors includes band 11q23. Cytogenetic and molecular studies both indicate that the 11q22-ter region contains a critical target for DNA damage in melanoma. Large-scale surveys of chromosome damage in melanomas have revealed a highly significant clustering of both chromosome loss and translocation breakpoints in 11q22-ter (6, 7). LOH in melanoma

Table 2 Growth properties of 903(11n) microcell hybrids

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Parental cells</th>
<th>Microcell hybrids</th>
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<tr>
<td></td>
<td>UACC 903</td>
<td>903(11n)1</td>
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<td>903(11n)2</td>
<td>903(11n)4</td>
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<td>28</td>
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<tr>
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*a Defined as the number of cells plated which formed macroscopic colonies after 3 weeks of incubation.
*b Latent period for tumor formation was defined as the number of days required for the mean tumor size to reach 100 mm³.

LOH in melanoma has been reported on 11q in several studies (11, 22, 23), and the most recent detailed investigation has defined a minimal region of LOH between the markers D11S33 and D11S968, a span of 51 cM centered at the APOC3 locus in band 11q23 (10).

Similar patterns of chromosome damage and LOH in chromosome bands 11q22–24 are emerging from genetic studies of other human solid tumors, including breast cancer (24–29), ovarian cancer (30–32), lung cancer (33, 34), stomach cancer (35), colon cancer (36), and cervical cancer (37–39). For breast, lung, and cervical cancers, this structural evidence has been bolstered by experiments which demonstrated suppression of tumorigenicity following microcell-mediated transfer of chromosome 11 into mammary, lung, and cervical carcinoma cell lines (40–42). In microcell hybrids derived from the HeLa cervical carcinoma cell line analysis of the loss of chromosome 11 polymorphic markers in tumorigenic revertants localized the suppressor locus to the 11q13–23 region (43).

The observation that hybrid 903(11n)2 formed tumors despite the presence of one intact chromosome 11 indicates that a single copy of the suppressor locus is insufficient to suppress tumor formation entirely. A very similar conclusion has recently been drawn in a recent study of chromosome 11 dosage effects on tumorigenicity in tumorigenic and nontumorigenic subclones of the HeLa D98-OR human cervical carcinoma cell line (21). It also remains possible that more than one gene is responsible for the suppression of cell growth and tumorigenicity seen in 903(11n) microcell hybrids. This seems unlikely since the two effects were correlated in all three of the hybrids, and the degree of growth suppression observed in vitro was sufficient to account for the effect on tumor formation. However, a more precise physical definition of the locus will be required to settle this question.

No specific genes on chromosome 11q have yet been put forward as candidates for the tumor suppressor involved in melanoma. The genes responsible for multiple endocrine neoplasia type 1 and ataxia telangiectasia are located at bands 11q13 and 11q23, respectively, but predisposition to melanoma is not a feature of either of these cancer susceptibility syndromes (44, 45). The short arm of chromosome 11 contains two known tumor suppressor loci involved in Wilms’ tumor, WT1 and WT2 (46), and the 11p arm suppresses the tumorigenicity of rhabdomyosarcoma cells (20, 47). But there is no evidence for preferential involvement of the 11p arm in melanoma chromosome abnormalities (6, 7) or for any significant role of the WT1 gene product in melanoma (48). Nor has any evidence been reported for linkage of familial melanoma to markers on chromosome 11q (23).

Additional efforts to refine the localization of the suppressor locus by means of physical mapping techniques will thus be required. Since there is some evidence that the presence of chromosome 11 abnormalities in melanoma predicts a poorer clinical outcome (12), the identification of the suppressor gene on this chromosome promises to provide a tool of considerable value for the physician. It may also have relevance to several other highly prevalent human cancers.
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