ABSTRACT

Malignant melanoma has been documented to display recurring abnormalities of chromosome 6, particularly the long arm (6q). Restriction fragment length polymorphism analysis was used as a molecular genetic approach to examine loci on chromosome 6q for loss of constitutional heterozygosity (LOH). Five DNA markers that recognize restriction fragment length polymorphisms along 6q and one polymorphic DNA marker for 6p were used to screen 20 autologous pairs of tumor DNA and normal DNA to determine the tumor and constitutional genotypes of each patient. LOH on chromosome 6q was identified at 21 of 53 informative loci (40%). Five patients with more than one informative locus had allele losses consistent with the loss of the entire long arm (or an entire copy) of chromosome 6, while four other patients demonstrated terminal deletions of 6q. The chromosomal region bearing the highest frequency of 6q allelic loss (60%) is defined by the marker loci c-MYB and ESR (6q22-23 and 6q24-27). In contrast to the frequency of 6q loss, LOH was observed at loci on four other chromosomes (1, 11, 16, 17) in only 5% of cases. These results have led us to conclude that the loss of sequences from the long arm of chromosome 6 is a nonrandom and possibly biologically relevant event in human malignant melanoma.

INTRODUCTION

The involvement of tumor suppressor genes in malignant transformation has been well documented (1-4). The mechanisms by which tumor suppressor genes contribute to the establishment and/or progression of tumor development involve the loss or inactivation of their wild-type alleles. Detection of this loss of somatic alleles (or LOH) in the tumor DNA of cancer-affected individuals has been possible through the use of polymorphic DNA markers. Nonrandom LOH has now been observed in many human neoplasms including retinoblastoma (5, 6), Wilms’ tumor (7, 8), breast carcinoma (9, 10), and glioblastoma multiforme (20).

Malignant melanoma is an increasingly important cancer whose rate of increase in incidence is second only to lung cancer in females (21, 22). To date, little is known of the genetic alterations underlying this important cancer; therefore, additional analyses are needed. Cytogenetic analysis of melanoma tumor cells reveals that approximately 40% of these tumors show deletions of the long arm of chromosome 6 (6q-) (23-25). In addition, we have recently demonstrated that the introduction of a normal copy of chromosome 6 into the genomes of melanoma cell lines (via microcell-mediated chromosome transfer) results in the loss of tumorigenicity of melanoma cells when injected into nude mice (26). Finally, limited data concerning LOH exists for melanoma with the overwhelming majority of studies performed to date on cell lines (27-29). However, no report of allelic loss on the long arm of chromosome 6 has been reported in any report of malignant melanoma.

In this study, we used RFLP analysis to investigate the frequency of loss of somatic sequences from the long arm of chromosome 6 in a group of 20 patients with histologically confirmed malignant melanoma, by screening pairs of tumor and normal DNA from each patient using five DNA markers that detect RFLPs on the long arm of chromosome 6 and one DNA marker from the short arm. In addition, the paired DNA samples were also screened with 4 VNTR polymorphic DNA markers from other chromosome loci, in order to confirm the specificity of chromosome 6 allelic losses.

MATERIALS AND METHODS

DNA Extraction. High molecular weight DNA was extracted from 20 metastatic tumor biopsies obtained from patients with histologically confirmed malignant melanoma and from autologous peripheral blood lymphocytes by lysis in a buffer containing 20 mM Tris-HCl, pH 8.0-10 mM EDTA-0.5% SDS-100 mM NaCl-10 µg/ml proteinase K at 55°C overnight. The lysate was extracted with phenol and chloroform and then ethanol precipitated. The DNA pellet was rinsed in 70% ethanol, dried, and resuspended in buffer (10 mM Tris-HCl, pH 8.0-1 mM EDTA). The prepared DNA was then treated with RNase A, reextracted with phenol and chloroform, and ethanol precipitated in the presence of sodium chloride. The final DNA pellet was rinsed with 70% ethanol, dried, and resuspended in buffer (10 mM Tris-HCl, pH 8.0-1 mM EDTA).

Southern Transfer. Genomic DNA (5 µg) from peripheral blood lymphocytes or tumor tissue were completely digested with a specific restriction enzyme, and the fragments were separated through 1.0% agarose gel electrophoresis. The DNA in the gels was alkali denatured overnight. The DNA was then transferred to nylon filters by the method of Southern (30). After transfer, the blots were neutralized in 0.5 mM Tris-HCl, pH 7.0-0.6 mM sodium chloride and dried in a vacuum oven at 80°C for 2 h.

Hybridization. The DNA probes (30-60 ng) were radioactively labeled with α-32P by the randomly primed oligonucleotide method (31) to a specific activity >106 cpm/µg and added to prehybridized filters in a hybridization solution containing 0.6 mM sodium chloride-8 mM EDTA-120 mM Tris-HCl, pH 7.4), 0.1% sodium pyrophosphate, 1.0% SDS, 10% dextran sulfate, 100 µg/ml denatured salmon sperm DNA. After 24 h at 65°C, the filters were washed in 0.1x standard sodium citrate-1% SDS at 56°C and exposed to X-ray film for autoradiography.

DNA Markers. The chromosome 6-specific polymorphic DNA markers and restriction enzymes used in the RFLP analysis are the following: pHM2.6 (c-myc), EcoRI; pJCZ30 (D6S37), EcoRI; pPMmsO2 (SOD2), TaqI; pEGalpa (CGA), EcoRI; pOR3 (ESR), PvuII; and pHHH157 (D6S29), BamHI. The polymorphic VNTR DNA markers for other non-chromosome 6 loci are: pYNZ22.1 (D17S30), TaqI; pYNZ22.1 (D17S30), TaqI; pJ3988 (H-ras), TaqI; and p97-2-23 (D16S57), TaqI.
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All chromosomal localizations and a description of the above marker loci are reported in the Human Gene Mapping 10 (32) and 10.5 proceedings (33). The probe D6S37 was sublocalized by hybridization to genomic DNA from a panel of somatic cell hybrids with chromosome 6 (34).

Cytogenetic Analysis. Metaphase preparation and G-banding of tumor chromosomes were performed as previously described (35).

RESULTS

We analyzed pairs of normal DNA (peripheral blood lymphocytes) and autologous tumor DNA (metastatic melanoma) from 20 melanoma patients using a panel of five polymorphic DNA markers localized to the long arm of chromosome 6 and one polymorphic marker localized to the short arm of 6. Fig. 1 shows three representative autoradiograms documenting LOH detected in melanoma tumor samples. In some cases, faint bands (corresponding to the deleted allele) could be visualized in the tumor sample representing traces of DNA from normal tissue infiltrating the tumor specimen.

The constitutional (normal) and tumor genotypes of all 20 melanoma patients at all five chromosome 6 loci are listed in Table 1. Tumors showing LOH at a given locus and their autologous normal genotype are indicated in solid boxes. In four cases allele loss could be identified in constitutionally homozygous patients, and these are indicated with dashed-line boxes in Table 1. These losses were confirmed by densitometric analysis of the hybridization signals when compared to other probes used as controls for DNA loading.

Fig. 2 is a schematic representation of the LOH data at all 6 loci from chromosome 6 in the 20 melanoma tumors studied. Chromosomal localization of each marker locus is represented with a solid bar. It can be observed from the data shown in Fig. 2 that, for each 6q marker locus analyzed, three informative patients (G. Z., K. Y., and P. R.) showed loss of allelic sequences, while no such losses were observed at the 6p locus. Furthermore, 6 patients (H. Y., H. N., K. Y., R. Z., S. H., and Z. O.) had allelic losses at every informative 6q locus, consistent with the loss of either an entire copy of chromosome 6 or the entire long arm. Patient K. Y., for instance, was heterozygous (informative) at the 6p locus, indicating preferential loss of the long arm of 6. RFLP analysis of patients H. Y., H. N., R. Z., and S. H. at the 6p marker locus was not possible. However, cytogenetic analysis of tumor cells from patient H. N. revealed loss of an entire copy of chromosome 6. In addition to whole chromosomal loss, terminal deletions of the long arm of chromosome 6 were observed in patients C. O., G. Z., K. T., and R. R.

Table 2 summarizes the frequency of LOH at the five 6q loci analyzed in our study. A total of 53 of 95 loci analyzed on the long arm of chromosome 6 were informative. Reduction to homozygosity/hemizygosity was observed in 21 of 53 inform-
## LOH FOR CHROMOSOME 6 IN MELANOMA

### Table 1 List of constitutional and tumor genotypes of 20 melanoma patients at six loci on chromosome 6

<table>
<thead>
<tr>
<th>Locus</th>
<th>CGA</th>
<th>D6S37</th>
<th>SOD2</th>
<th>c-MYB</th>
<th>ESR</th>
<th>D6S29</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pcGalphi</td>
<td>pJCZ30</td>
<td>pHMnSOD4</td>
<td>pHM2.6</td>
<td>pOR3</td>
<td>pHHH157</td>
</tr>
<tr>
<td>6q12-21</td>
<td>6q13-21</td>
<td>6q21</td>
<td>6q22-23</td>
<td>6q24-27</td>
<td>6p</td>
<td></td>
</tr>
<tr>
<td>EcoR1</td>
<td>EcoR1</td>
<td>TaqI</td>
<td>EcoR1</td>
<td>Prull</td>
<td>BamHII</td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td>Specimen*</td>
<td>A1/A2</td>
<td>A2</td>
<td>A2</td>
<td>A2</td>
<td>A1/A2</td>
</tr>
<tr>
<td>A. N.</td>
<td>N</td>
<td>T</td>
<td>A1/A2</td>
<td>A1</td>
<td>A2</td>
<td>A2</td>
</tr>
<tr>
<td>M. G.</td>
<td>N</td>
<td>T</td>
<td>A1/A2</td>
<td>A2</td>
<td>A1/A2</td>
<td>A2</td>
</tr>
<tr>
<td>P. S.</td>
<td>N</td>
<td>T</td>
<td>A1/A2</td>
<td>A2</td>
<td>A1/A2</td>
<td>A2</td>
</tr>
<tr>
<td>S. O.</td>
<td>N</td>
<td>T</td>
<td>A1/A2</td>
<td>A2</td>
<td>A1/A2</td>
<td>A2</td>
</tr>
<tr>
<td>V. M.</td>
<td>N</td>
<td>T</td>
<td>A1/A2</td>
<td>A2</td>
<td>A1/A2</td>
<td>A2</td>
</tr>
<tr>
<td>V. A.</td>
<td>N</td>
<td>T</td>
<td>A1/A2</td>
<td>A2</td>
<td>A1/A2</td>
<td>A2</td>
</tr>
</tbody>
</table>

* N, normal; T, tumor.

![Fig. 2. Schematic representation of LOH at 6 loci on chromosome 6 for 20 melanoma patients. Top abscissa, patient codes. Allele loss in informative cases (●), no loss in informative cases (●), or homozygous noninformative (○) cases are shown aligned below each patient.](image)

- no information
- informative - no loss
- non-informative
- informative - loss

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In an attempt to relate the frequency of LOH on 6q in melanoma to other genomic sites, we have also examined the frequency of LOH on other chromosomes (1p, 11p, 16q, and 17p) using a series of highly informative VNTR probes. Sixty-five loci were analyzed with these non-chromosomal probes. Of these, 54% of 40 informative loci showed LOH (5%).

Previous studies by Dracopoli et al. (27) utilizing cell lines established from melanoma metastases failed to show any specificity of chromosomal LOH. However, no loci from the long arm of chromosome 6 were included in this study. Likewise, Nordenskjöld et al. (28) recently reported a random pattern of allele loss in a group of 11 metastatic melanoma tumors analyzed using a panel of polymorphic probes. However, as in the study of Dracopoli et al. (27), the probe recognizing a polymorphism on chromosome 6 was localized to the short arm. Finally, although the study of Orita et al. (29) did utilize a 6q probe (MYB), only 2 of 8 patients were informative at this loci and neither documented LOH. Thus, this current study represents the initial report for malignant melanoma documenting LOH for the long arm of chromosome 6.

In our current studies of 20 metastatic melanoma tumors we have revealed a significant (40%) and apparently nonrandom pattern of somatic allele losses from the long arm of chromosome 6. In about half of the tumors analyzed, the mechanism for LOH appeared associated with whole chromosome loss (e.g., via nondisjunction); while in the remaining cases, specific loss of the long arm was documented by both LOH and cytogenetic analysis. The results of LOH reported here, together with the extensive cytogenetic (23–25) and new biological data (26), further support the notion that a tumor suppressor gene important in melanoma may be localized to the long arm of chromosome 6.

ACKNOWLEDGMENTS

We thank Dr. I. Boime for providing us with the polymorphic probe pCGalpha used in this study.

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Loss of Heterozygosity for Loci on the Long Arm of Chromosome 6 in Human Malignant Melanoma

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