

Loss of Heterozygosity for Loci on the Long Arm of Chromosome 6 in Human Malignant Melanoma¹

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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 of 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 LOH³) 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), Wilm's tumor (7, 8), breast carcinoma (9, 10), small cell lung carcinoma (11, 12), malignant astrocytoma (13, 14), neuroblastoma (15), colorectal carcinoma (16-18), meningioma (19), 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 in 0.4 M sodium hydroxide-0.6 M sodium chloride, prior to transfer to nylon filters by the method of Southern (30). After transfer, the blots were neutralized in 0.5 M Tris-HCl, pH 7.0-0.6 M 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 >10⁸ cpm/µg and added to prehybridized filters in a hybridization solution containing 0.6 M 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 0.1× 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-myb*), *EcoRI*; pJCZ30 (D6S37), *EcoRI*; pHMnSO₄ (SOD2), *TaqI*; pcGalpha (CGA), *EcoRI*; pOR3 (*ESR*), *PvuII*; and pHHH157 (D6S29), *BamHI*. The polymorphic VNTR DNA markers for other non-chromosome 6 loci are: pYNZ2 (D1S57), *TaqI*; pYNZ22.1 (D17S30), *TaqI*; pEJ988 (*H-ras*), *TaqI*; and p79-2-23 (D16S7), *TaqI*.

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³ The abbreviations used are: LOH, loss of constitutional heterozygosity; RFLP, restriction fragment length polymorphism; VNTR, variable number of tandem repeats; SDS, sodium dodecyl sulfate.

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/hemizyosity was observed in 21 of 53 inform-

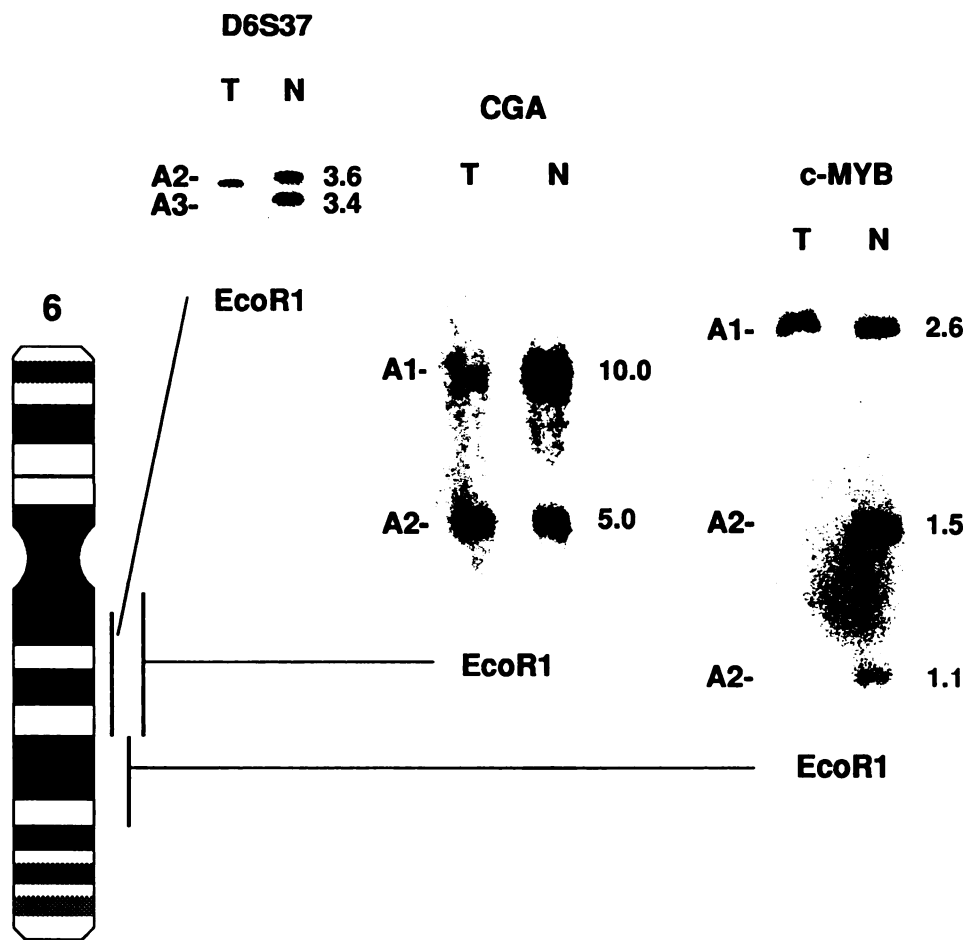
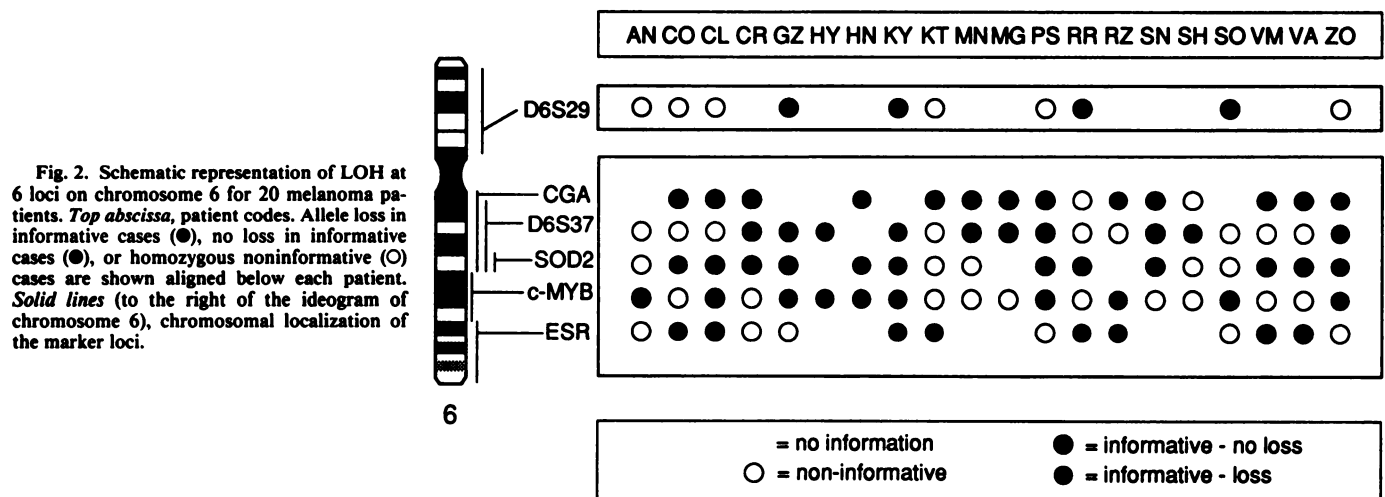


Fig. 1. Representative Southern blots of EcoRI-digested tumor (T) and autologous normal peripheral blood lymphocyte (N) DNA hybridized to polymorphic probes for the marker loci D6S37, CGA, and c-MYB. Heterozygosity for three different patients is detected in the constitutional (N) DNA shown in all panels; loss of one allele (LOH) is observed in all three tumors. Right ordinate (of autoradiograms), size of the alleles in kilobase pairs (Kb); solid lines (to the right of the chromosome 6 ideogram), chromosomal localization of each probe.

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

Patient	Specimen ^a	Locus					
		CGA	D6S37	SOD2	c-MYB	ESR	D6S29
		Probe					
		pcGalpha	pJCZ30	pHMnSOD4	pHM2.6	pOR3	pHHH157
		Localization					
6q12-21	6q13-21	6q21	6q22-23	6q24-27	6p		
Restriction enzyme							
		EcoRI	EcoRI	TaqI	EcoRI	PvuII	BamHI
A. N.	N		A2	A1	A1/A2	A2	A1
	T		A2	A1	A1/A2	A2	A1
C. O.	N	A1/A2	A2	A1/A2	A2	A1/A2	A1
	T	A1/A2	A2	A1	A2	A1	A1
C. L.	N	A1/A2	A3	A1/A2	A1/A2	A1/A2	A1
	T	A1/A2	A3	A1/A2	A1/A2	A1/A2	A1
C. R.	N	A1/A2	A2/A3	A1/A2	A2	A2	
	T	A1/A2	A2/A3	A1/A2	A2	A2	
G. Z.	N		A1/A3	A1/A2	A1	A2	A1/A2
	T		A1/A3	A1/A2	A1	A2	A1/A2
H. Y.	N		A2/A3		A1		
	T		A3		A1		
H. N.	N	A1/A2		A1/A2	A1/A2		
	T	A1		A1	A1		
K. Y.	N		A1/A3	A2	A1	A1/A2	A1/A2
	T		A3	A2	A1	A1/A2	A1/A2
K. T.	N	A1/A2	A1	A2	A2	A1/A2	A1
	T	A1/A2	A1	A2	A2	A1/A2	A1
M. N.	N	A1/A2	A1/A3	A2	A1		
	T	A1/A2	A1/A3	A2	A1		
M. G.	N	A1/A2	A2/A3		A2		
	T	A1/A2	A2/A3	A2			
P. S.	N	A1/A2	A2/A3	A1/A2	A1/A2	A2	A2
	T	A1/A2	A2/A3	A1/A2	A1/A2	A2	A2
R. R.	N	A1	A1	A1/A2	A2	A1/A2	A1/A2
	T	A1	A1	A1/A2	A2	A1	A1/A2
R. Z.	N	A1/A2	A2		A1/A2	A1/A2	
	T	A1	A2		A2	A2	
S. N.	N	A1/A2	A2/A3	A1/A2	A2		
	T	A1/A2	A2/A3	A1/A2	A2		
S. H.	N	A1	A2/A3	A1	A2		
	T	A1	A3	A1	A2		
S. O.	N		A2	A1	A1/A2	A2	A1/A2
	T		A2	A1	A1/A2	A2	A1/A2
V. M.	N	A1/A2	A2	A1/A2	A2	A1/A2	
	T	A1/A2	A2	A1/A2	A2	A1/A2	
V. A.	N	A1/A2	A2	A1/A2	A2	A1/A2	
	T	A1/A2	A2	A1/A2	A2	A1/A2	
Z. O.	N	A1/A2	A2/A3	A1/A2	A1/A2	A1	A2
	T	A2	A2	A2	A1	A1	A2

^a N, normal; T, tumor.



ative loci for 6q, representing an overall frequency of allelic loss of 40%.

For the purpose of determining the specificity of LOH for loci on the long arm of 6, we hybridized our Southern blots with polymorphic (VNTR) probes localized to chromosomes 17p(pYNZ22.1), 1p(pYNZ2), 11p(pEJ988), and 16q(79-2-23). The results from this RFLP analysis are summarized in Table 3. It can be observed that, of 39 informative (heterozygous) loci, 37 retained their constitutional genotype, while only 2 showed allelic loss. This represents an overall frequency of LOH of only 5% for these four other chromosome loci. The frequency and nonspecificity of LOH for these four loci are consistent with other recent reports for melanoma which describe an apparent random pattern of LOH in this disorder (27-29), further suggesting a significant role for 6q loss in melanoma.

DISCUSSION

LOH for specific chromosome loci is potentially indicative of somatic mutations which may be important in the establishment or progression of malignancy. In this study, we analyzed 20 paired DNA samples obtained from histologically confirmed melanoma tumor biopsies and from peripheral blood lymphocytes. The constitutional and tumor genotypes of each patient at five chromosome loci on the long arm of chromosome 6 [CGA(6q12-21), D6S37(6q13-21), SOD2(6q21), c-Myb(6q22-23), and ESR(6q24-27)] were examined. In addition, the normal and tumor genotypes of 10 of these patients were examined using a polymorphic marker (D6S29) which maps to the short arm of chromosome 6. Our results demonstrate that LOH for the long arm of chromosome 6 is a very frequent event occurring in 40% (21 of 53) of informative loci. In contrast, no examples of LOH were detected for the short arm of this chromosome. These observations are in good general agreement with the cytogenetic data concerning melanoma which has indicated that, while the loss of the long arm of chromosome 6 is a common observation in melanoma (23-25), 6p remains unchanged or is in fact duplicated [usually as an iso(6p)] in this disorder (36).

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 four other chromosomal loci (1p, 11p, 16q, and 17p) using a series of highly informative VNTR probes. Sixty-five loci were analyzed with these non-chromosome 6 probes with only 2 of 39 informative loci showing 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,

Table 2 Frequency of LOH for five loci on the long arm of chromosome 6 in malignant melanoma

Marker locus	Chromosome region	No. of cases studied	No. of informative cases	No. of cases with LOH	LOH % of informatives
CGA	6q12-21	15	13	3	23
D6S37	6q13-21	19	10	4	40
SOD2	6q21	17	12	4	33
c-MYB	6q22-23	20	10	6	60
ESR	6q24-27	14	8	4	50
Totals (average)		95	53	21	(40)

Table 3 Frequency of LOH on chromosomes 1, 11, 16, and 17 in malignant melanoma

Probe	Chromosome region	No. of cases studied	No. of informative cases	No. of cases with LOH	LOH % of informatives
pYNZ2	1p	17	12	0	0
pEJ988	11p	17	10	1	10
79-2-23	16q	14	10	0	0
pYNZ22.1	17p	17	7	1	14
Totals (average)		65	39	2	(5)

Nordenskjold *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.

Our LOH studies of 20 metastatic melanoma tumors 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.

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