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Advances in Brief

Loss of Heterozygosity Analysis Defines a Critical Region in Chromosome 1p22 Commonly Deleted in Human Malignant Mesothelioma[1]

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Abstract

Previous cytogenetic analysis has revealed frequent losses of chromosome 1p21-22 in human malignant mesothelioma, suggesting that the loss or inactivation of a tumor suppressor gene(s) residing at this site may contribute to the tumorigenic conversion of mesothelial cells. To more precisely define the location of the target gene, primary tumor specimens and cell lines from 50 malignant mesotheliomas were examined for loss of heterozygosity using short tandem repeat polymorphism (STRP) markers. Nineteen STRP markers established by the Cooperative Human Linkage Center were selected for the initial screening of the entire short arm of chromosome 1. Thirty-seven cases (74%) showed allelic losses at least at one locus in 1p. Thirty-six of these cases showed losses of 1p21–22, including 23 with partial deletions involving this region. To obtain a higher resolution map of this region, another 13 STRP markers from the Genethon map were used to define the shortest region of overlapping deletions to a 4-cM segment flanked by the loci *D1S435* and *D1S236*. The chromosomal location of the critically deleted region was confirmed to be within 1p22 by karyotypic and fluorescence *in situ* hybridization analyses.

Introduction

Tumorigenesis is a multistep process involving a series of acquired genetic changes, including activation of proto-oncogenes and inactivation of TSGs.[3] Some of these changes involve gross alterations of chromosomal structure visible as cytogenetic abnormalities. In fact, the association between recurrent chromosomal abnormalities and specific types of cancer has led to the discovery of genes which, when altered, contribute to the initiation or progression of human malignancy (reviewed in Ref. 1).

MM is a neoplasm of mesodermal origin with an incidence of approximately 2000 cases annually in the United States. Exposure to asbestos has been implicated as a major contributory factor in the development of this malignancy. MM is characterized by a very long latency from the time of exposure to asbestos to the onset of disease. The length of the latent period suggests that multiple genetic alterations may be required for tumorigenic conversion of a mesothelial cell. This notion is supported by previous karyotypic analyses demonstrating that most MMs have multiple numerical and structural changes (2–4). Recurrent deletions of specific chromosomal sites in MM include 1p21–22, 9p21–22, 3p21, 6q15–21, and 22q (4), suggesting that TSGs critical to mesothelial cell tumorigenesis may reside in these regions. At the molecular genetic level, we have detected high frequencies of LOH at 3p21 in MM tumor biopsies and cell lines (5) and homozygous deletions at 9p21–22 in MM cell lines (6). We further demonstrated that *CDKN2/MTS1/p16,* located at 9p21–22, is homozygously deleted in many MM cell lines in addition to several tumor specimens (7). Moreover, *NF2,* a TSG located in chromosome 22q12, has been shown to be a frequent site of mutations in primary tumors and cell lines from patients with MM (8, 9).

In this study, the high frequency of cytogenetic deletions within 1p in MM was verified at the molecular genetic level using STRP markers. We report the results of high resolution deletion mapping of 1p using 32 cell lines and 18 tumor specimens and fine map the SRO of these deletions to a region of approximately 4 cM. In addition, we confirm the location of the SRO in 1p22 by karyotypic and FISH analyses.

Materials and Methods

Specimen Collection and Nucleic Acid Extraction. Criteria for the diagnosis of MM were in accordance with established guidelines (10). All primary MM tumors were snap-frozen and stored at −70°C prior to extraction of DNA. Thirty-two cell lines were established from surgically explanted primary MMs, as described previously (4). Genomic DNAs were purified from the cell lines and tumors by standard methods (6) and from matched blood samples with the aid of a QIAamp kit (Qiagen).

PCR and LOH Studies. All primer pairs for PCR amplification of STRP markers were obtained from Research Genetics (Huntsville, AL). Each PCR was performed in a 10-μl mixture containing 30 ng of genomic DNA, 0.4 μM of each primer, 0.64 μM dATP, 4 μM each of dCTP, dTTP and dGTP, 1 μCi of [α-^{35}S]dATP (DuPont NEN), and 0.4 unit of *Taq* DNA polymerase in GeneAmp PCR buffer (Perkin-Elmer). For most of the primer pairs, the mixtures were first heated to 94°C for 4 min and then subjected to 35 PCR cycles, each consisting of 94°C for 30 s, 57°C for 30s, and 72°C for 30s, with a final 5-min extension at 72°C. To improve the results obtained with some primer pairs, the annealing temperature was lowered to 55°C, and the extension time was increased to 1 min. Five μl of each PCR product was separated on 6% polyacrylamide sequencing gels. Gels were dried and then exposed to Kodak X-AR5 films for visualization. To determine band intensity differences, some dried gels were also exposed to a phosphorimaging plate and quantitated by a Fuji Bio Imaging analyzer.

Fifty cases, including 18 tumor specimens and 32 cell lines, from primary MMs were included in this study. Genomic DNA from matched blood samples was used for comparison in all of the tumor cases and 19 of the cell lines. DNA isolated from tumor explants was used to determine the constitutional heterozygosity for the other 13 cell lines (5), because no matched blood samples were available in these cases. For all cell lines, LOH was assigned to an informative locus when one allele was completely lost in the cell line. For tumor tissues, LOH was assigned if the intensity of the band from one allele was greatly reduced as compared to its corresponding normal allele. However, if the tumor DNAs showed less marked differences in intensity of allelic bands, the signals were quantitated by phosphorimaging, and LOH was assigned if the allelic ratio in tumor DNA differed more than 30% from the allelic ratio in normal DNA.

Cytogenetic and FISH Analyses. Cell line 28 was used for both karyotypic and FISH studies. Actively growing cells were harvested, fixed, and

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[3] The abbreviations used are: TSG, tumor suppressor gene; MM, malignant mesothelioma; LOH, loss of heterozygosity; STRP, short tandem repeat polymorphism; SRO, shortest region of overlap; FISH, fluorescence *in situ* hybridization; CHLC, Cooperative Human Linkage Center; YAC, yeast artificial chromosome.

G-banded according to standard methods (4). Ethidium bromide was added 2 h before cytogenetic harvest to extend the length of chromosomes available for banding analysis (11).

Yeast strains carrying YACs 885C5, 946C5, 957D9, and 759D7 (containing 1p loci *D1S488, D1S435, D1S424,* and *D1S236,* respectively) were obtained from Research Genetics. DNA was purified from yeast strains by standard methods. Two-color FISH was performed using biotin-16-dCTP and digoxigenin-11-dUTP. Probes were labeled by random octamer priming at 37°C for 1 h and then ethanol coprecipitated with Cot1 DNA. Hybridization was carried out on metaphase spreads from phytohemagglutinin-stimulated normal peripheral blood lymphocytes and cells from case 28, as described previously (12). Hybridization of biotinylated probes was detected with FITC-avidin (Oncor, Gaithersburg, MD) and digoxigenin-labeled probes with anti-digoxigenin rhodamine (Boehringer Mannheim). Chromosomes were counterstained with 4′,6′-diamidino-2-phenylindole. Images of 4′,6′-diamidino-2-phenylindole-stained chromosomes and hybridization signals were captured separately using a cooled CCD camera (Photometrics, Tucson, AZ), pseudo-colored, and then merged using computer imaging software from Oncor.

Results

Detection of LOH in MM by STRP Analysis. To map the region of deletion in 1p, 32 cell lines and 18 tumor specimens were examined for LOH using STRP markers. Nineteen CHLC markers (13) were selected for the initial screening. Seventeen of these STRP markers, distributed along the entire short arm (Fig. 1), were used to delineate deletions in 1p, and two others, located very close to the centromere in the long arm of chromosome 1, were used to determine if deletions extended into 1q, as would be the case for whole chromosome losses and other pericentric losses. All 50 cases were informative at several of the tested loci (range, 7–16; median, 12.5). The highest frequency of deletion was at *D1S1588* (20 of 26 informative cases, 77%; Fig. 1). Frequent LOH was also observed at *D1S1587* (21 of 29 informative cases, 72%) and *D1S551* (21 of 31 informative cases, 68%), which flank the *D1S1588* locus. The marker GCT1C9 also showed a high frequency (70%) of LOH, but only 10 cases were informative with this marker.

Overall, 37 cases (74%) showed LOH at least at one locus. Thirteen of these 37 cases (8 cell lines and 5 tumors) lost alleles at loci spanning the entire short arm of chromosome 1, and the loss extended into the long arm in 10 cases (6 cell lines and 4 primary tumors). These cases could not be used to narrow down the critically deleted region since an entire 1p or a whole chromosome 1 was lost. Interstitial and/or terminal deletions were observed in the remaining 24 cases, 23 of which involved 1p21–22 (Fig. 1). By analyzing the LOH patterns of the latter cases, the SRO of deletions was determined to be located between markers *D1S551* and *D1S1587*, consistent with the high frequency of LOH at these loci. Case 9 defined the proximal border since one allele at *D1S1588* and each informative locus distal to it was lost, but heterozygosity was retained at *D1S1587* and all of the 1p loci proximal to it (Figs. 1 and 2). Cases 54, 63, and 28 defined the distal boundary of the SRO. For example, case 28 showed LOH at *D1S1588* but retained heterozygosity at *D1S551*, the next distal locus, and all informative loci distal to it (Figs. 1 and 2). Only two cases (2 and 31) retained heterozygosity at *D1S1588*. In case 2, *D1S534* was the sole locus that showed allelic loss. However, the overall frequency of LOH at this locus was only 48%, and many cases with interstitial deletions were heterozygous at this locus; thus, this site may not represent another critically deleted region in MM. STRP analysis of case 31 showed that both alleles in the region from *D1S532* to *D1S1588* were retained, but karyotypic analysis of this cell line

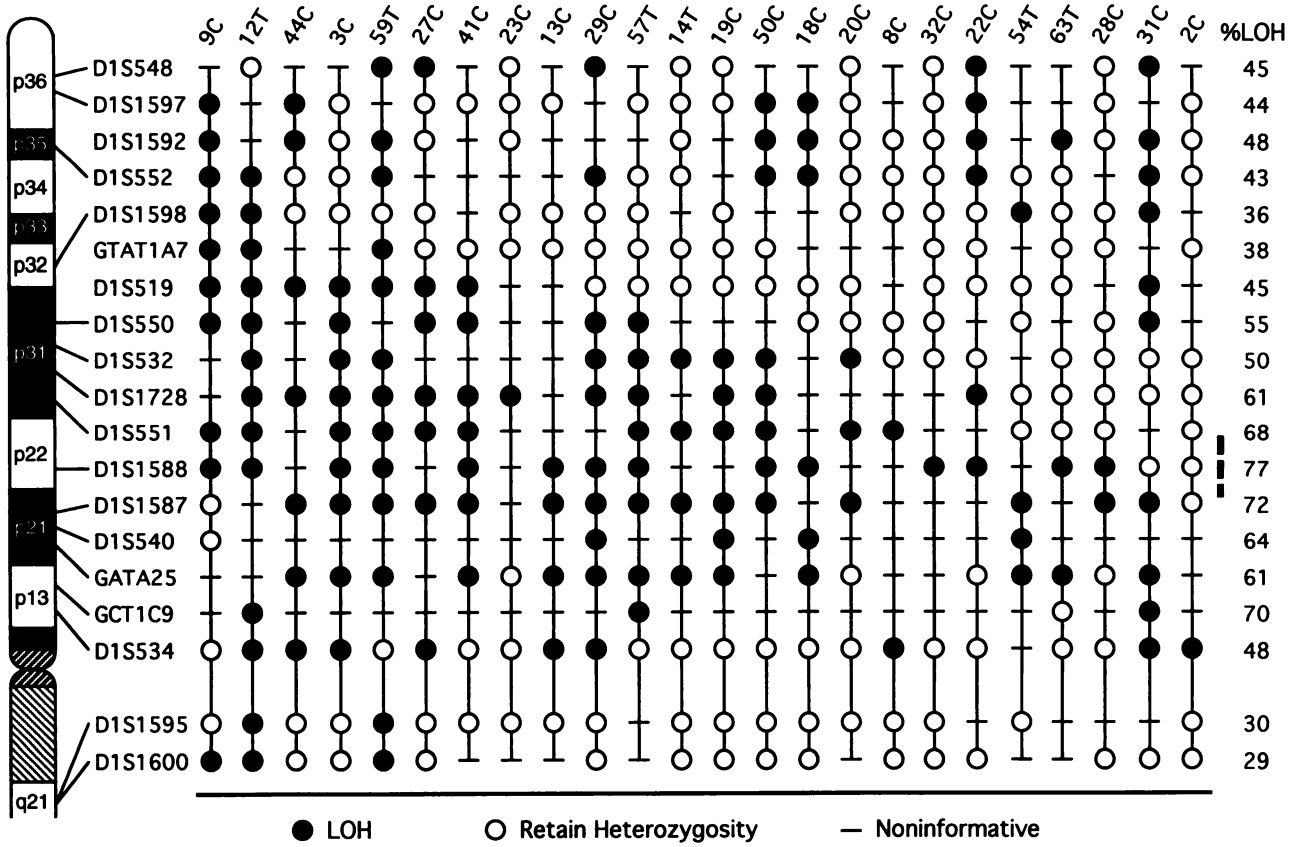

Fig. 1. LOH analysis of chromosome 1 at 19 CHLC markers in MM. The percentage of LOH (LOH cases/informative cases) at each locus in all 50 MM cases is shown at the *right*. The allelic losses in 24 cases with interstitial and/or terminal deletions are presented. Case numbers are indicated at the *top*. Tumor (*T*) or cell line (*C*) was used for LOH analysis. The SRO of deletions is indicated at the *right* by a *vertical broken line*.

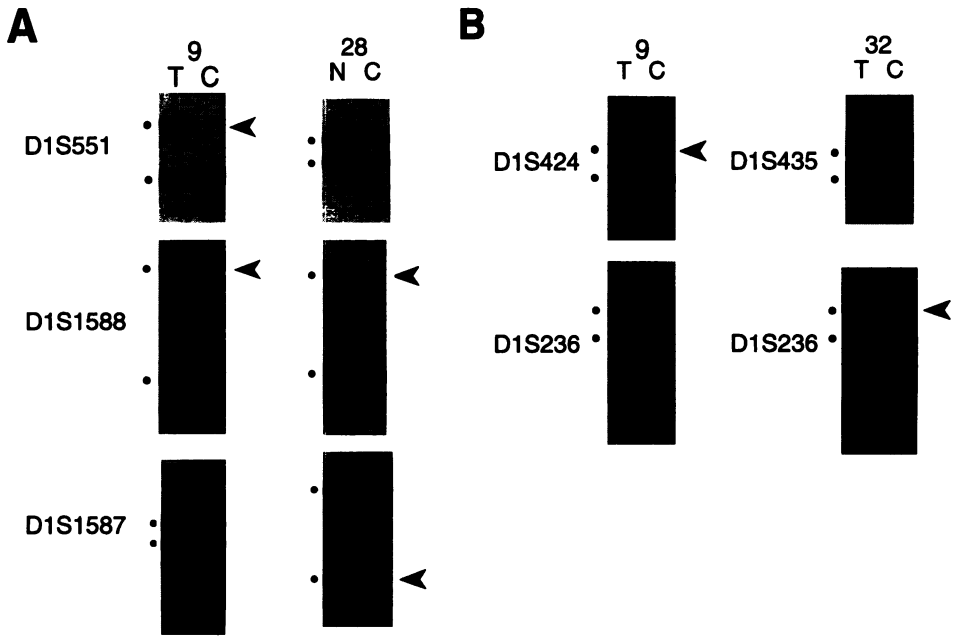

Fig. 2. Representative autoradiographs of STRP analyses that define the SRO of 1p deletions in MM. *A*, CHLC markers; *B*, Genethon markers. Case numbers are shown at the *top*. *T*, tumor DNA; *C*, cell line DNA; *N*, normal lymphocyte DNA. *Dots*, positions of the alleles; *arrowheads*, allelic loss.

indicated that only one copy of chromosome 1 was present (data not shown), suggesting that a relatively small segment of 1p resides in one of the marker chromosomes observed in this cell line. One of the breakpoints in case 31 is located between *D1S1588* and *D1S1587*, further implicating this region as being critical in MM.

To obtain a more refined map of the SRO of 1p deletions, another 13 STRP markers located near *D1S1588* were selected from the second generation Genethon map (14, 15). All 50 MMs were tested for these markers, but no new deletion was revealed. The LOH patterns in 8 cases with a breakpoint at either side of *D1S1588* were used to determine the SRO (Fig. 3). Cases 13 and 18 exhibited losses at all informative Genethon loci (data not shown). Through an analysis of the informative cases, the SRO can be delineated as a region of approximately 4 cM between *D1S435* and *D1S236*. The proximal boundary, *D1S236*, was determined by the location of the breakpoint in case 9 (Figs. 2 and 3). Case 32 was not informative for *D1S424* and *D1S415* but showed allelic losses at the proximal loci and retained heterozygosity at *D1S435* and more distal loci, suggesting the distal border to be *D1S435* (Figs. 2 and 3). Interestingly, in case 31 heterozygosity was retained at *D1S424* but was lost at *D1S415*. The complicated pattern of deletion in this case suggests that a second rearrangement, such as an inversion, might have occurred prior to the deletion event.

Cytogenetic Analyses. The location of the SRO at 1p22 was verified by two-color FISH analysis using YACs containing loci flanking the SRO. When YAC 946C5 (containing *D1S435*) and YAC 759D7 (containing *D1S236*) were hybridized to metaphase spreads of normal lymphocytes, both signals were observed on 1p22 (Fig. 4*A*), indicating that the SRO is located within this band. The two signals appeared to be very close to but distinct from each other, with YAC 946C5 signals located distal to those for YAC 759D7 in most copies of chromosome 1 examined. These observations are consistent with the relative orientation of *D1S435* and *D1S236* and the 4-cM distance inferred from the genetic map (Fig. 3).

Among all of the cases included in this study, cell line 28 appeared to contain the smallest interstitial deletion involving the region 1p21–22 (Figs. 1 and 3) and thus was used to identify the chromosomal location of the deletion by G-banding and FISH analyses. The modal chromosome number of this cell line was near-triploid, with

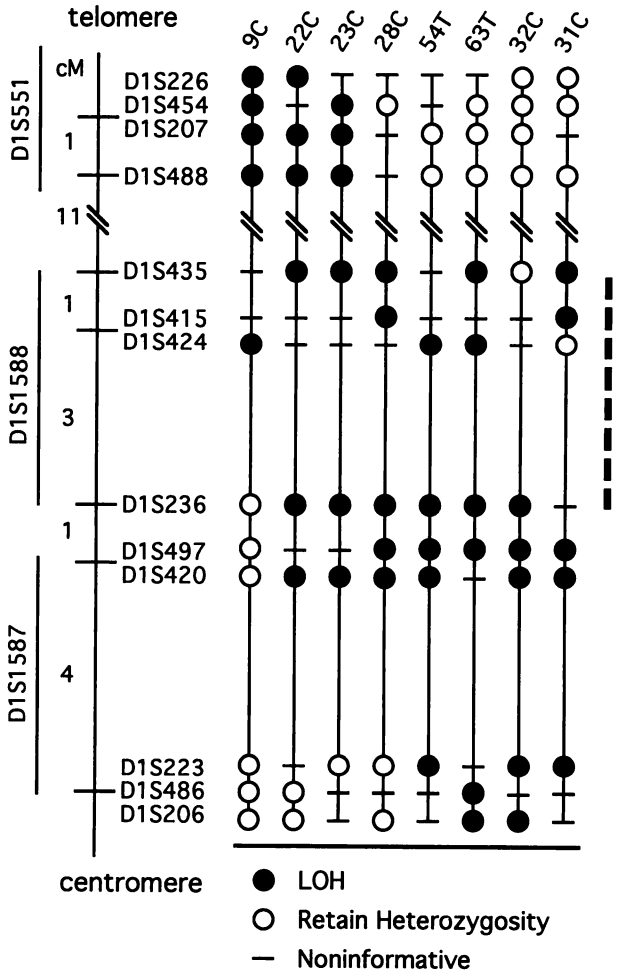

Fig. 3. LOH analysis at 13 Genethon markers in 8 MM cases used to determine the boundary of the SRO of 1p deletions. Case numbers are indicated at the *top*. Genetic distance in cM is based on the Genethon map (15). The most likely locations of three CHLC markers are represented by a *line* at the *left*. Tumor (*T*) or cell line (*C*) was used for determining the LOH pattern. The SRO is indicated by a *vertical broken line* at the *right*.

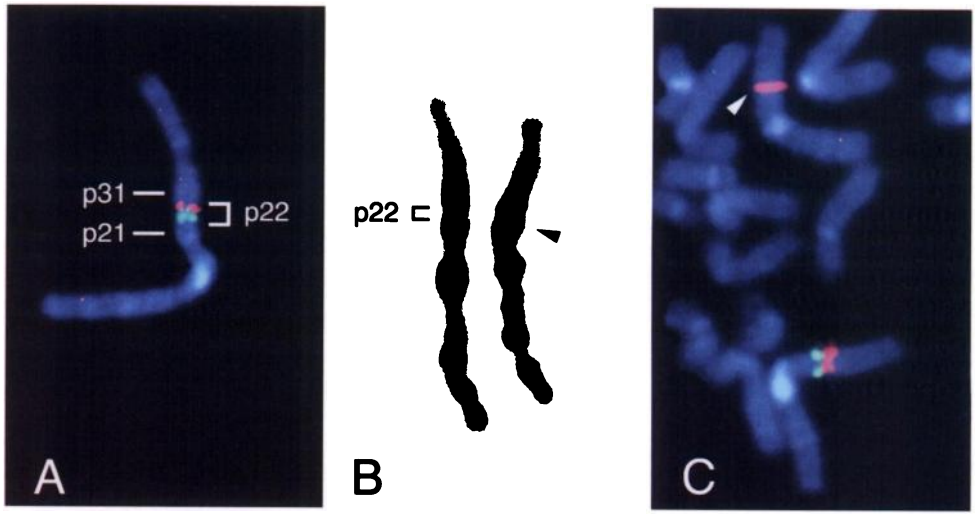

Fig. 4. Karyotypic and FISH analyses. *A*, two-color FISH analysis of a chromosome 1 from a normal lymphocyte metaphase spread. Rhodamine (*red*) signal: YAC 946C5 (containing marker *D1S435*); FITC (*green*) signal: 759D7 (containing *D1S236*). *B*, one normal and one aberrant copy of chromosome 1 from a metaphase of cell line 28. Note deletion of part or all of 1p22 (*arrowhead*). *C*, two-color FISH analysis of cell line 28. Rhodamine signals: 885C5 (containing *D1S488*); FITC signal: 957D9 (containing *D1S424*). *Arrowhead*, chromosome with interstitial deletion involving 1p22.

three copies of chromosome 1 in most metaphases. Two copies appeared to be normal. Band 1p22 appeared to be deleted in the remaining copy (Fig. 4*B*).

In a two-color FISH experiment performed on metaphase spreads from cell line 28, probes derived from YACs 957D9 (containing *D1S424*) and 885C5 (containing *D1S488*) hybridized to both normal copies of chromosome 1. The aberrant chromosome 1 hybridized to the 885C5 probe but not to the 957D9 probe (Fig. 4*C*), confirming the deletion encompassing locus *D1S424*.

Discussion

Deletion of chromosome 1p21–22 was the most frequent change observed in our previous cytogenetic analyses of primary MM tumor specimens and cell lines (4). In the study presented here, this deletion was verified at the molecular genetic level by LOH analysis using STRP markers spanning the entire short arm of chromosome 1. Moreover, the SRO of deletions was narrowed down to a 4-cM segment between the loci *D1S435* and *D1S236* by analyzing the densely distributed $(CA)_n$ markers located in this region. FISH analysis using YACs containing *D1S435* and *D1S236* confirmed that the critically deleted region resides within band 1p22.

Among the 50 cases included in this investigation, deletions in 1p were observed in 26 cell lines and 11 tumors. Tumor samples had a lower frequency (11 of 18, 61%) of 1p deletions than cell lines (26 of 32, 81%), which could be due to various factors, such as contamination of tumor tissue by normal infiltrating cells, confounding efforts to identify LOH. Also, our previous karyotypic studies documented heterogeneity within tumors from MM patients, and there could be *in vitro* selection of subpopulations exhibiting 1p deletions. In the majority of tumor specimens, LOH was easily recognized because the intensity of the band amplified from one allele of the tumor DNA was greatly reduced as compared to its corresponding allele in normal cells. However, PCR amplification products from some tumor DNAs showed less obvious differences in intensity. In this study, LOH was assigned to a locus only if the allelic ratios in tumor and normal DNAs differed more than 30%. Thus, an allelic loss in a tumor specimen could be overlooked if there was contamination by a large amount of infiltrating lymphocytes or cells from adjacent normal tissue. Nonetheless, interstitial deletions were detected in six primary tumor specimens, and the locations of the deletions were comparable to those of the cell lines. Moreover, karyotypic analysis also showed that both fresh tumors and cell lines shared the same characteristic deletions of the 1p21–22 region (4), further supporting the notion that the deletion events occurred before tumor explantation and were preserved in the cell lines.

Recurrent deletions involving 1p have been observed by cytogenetic and/or LOH analyses in several different cancers. Chromosome 1p36 is frequently deleted in neuroblastoma, malignant melanoma, Merkel cell carcinoma, colon cancer, hepatocellular carcinoma, and breast cancer (16). In this study, approximately 45% of MM cases showed LOH from 1p36 (Fig. 1). Although this frequency is considerably less than that observed at 1p22, the possibility exists that a TSG residing in 1p36 may also be involved in the pathogenesis of some MMs. Chromosome 1p22 was reported to be an unique deletion site associated with male germ cell tumors (17). There are no markers in common between the ones used in the present investigation and those used in the deletion mapping study of male germ cell tumors, and the relative locations between these two sets of markers are unknown. Thus, it is unclear at this time whether the same 1p22 TSG may be involved in both of these tumor types. It is also noteworthy that in a recent study of liver carcinomas, deletion of 1p, with a breakpoint at 1p22, was one of the most frequent abnormalities (18), but the critical region has not yet been defined molecularly. Several other regions in chromosome 1p have also been reported to be frequently deleted in different kinds of cancer, *e.g.*, 1p32–35 in neuroblastoma (16), 1p31.3–32.2 and 1p13 in male germ cell tumors (17), and 1p35, 1p31, and 1p13 in breast cancer (19, 20). The 1p31 deletion in breast cancer is quite close physically to the 1p22 deletion we observed in MM. However, the 1p31 markers most frequently deleted in breast cancer (20) are more than 10 cM distal to the SRO defined in our investigation, suggesting that different TSGs are involved in these two malignancies.

It has been well documented that multiple genetic changes contribute to the pathogenesis of various types of malignancies. In MM, the long disease latency and the recurrent nature of losses from several different chromosomes suggest that inactivation of multiple TSGs may be required for mesothelial cell tumorigenesis. Frequent alterations of two TSGs, *CDKN2* (*p16*) and *NF2*, have been observed in both MM cell lines and primary tumors (7–9). Mutations of other TSGs, such as *TP53* and *WT1*, have also been reported in a few cases of MM (21, 22). The deletion mapping data presented here not only strongly suggest that a putative 1p22 TSG(s) may be altered in a high percentage of MMs but also define its location to a discrete 4-cM region. With the rapid advances in human genome mapping and contig construction, it should now be possible to begin positional cloning of this region. We are currently in the process of constructing

a YAC contig encompassing the SRO. Such a contig will allow us to generate new polymorphic markers to define an even smaller SRO as well as search for candidate genes residing in this region.

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