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Neurofibromatosis Type 2 (*NF2*) Gene Is Somatically Mutated in Mesothelioma but not in Lung Cancer[1]

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Abstract

We have found 16 of 28 small cell lung cancers, 17 of 31 non-small cell lung cancers, 2 of 3 carcinoids, and 12 of 14 mesotheliomas that had chromosome 22 cytogenetic abnormalities. To determine whether the neurofibromatosis type 2 (*NF2*) gene located on chromosome 22 participates in the oncogenesis of these malignancies, we studied DNAs from lung cancer cell lines and mesotheliomas using Southern blot analysis and the single-strand conformation polymorphism (SSCP) technique for mutations covering 8 of the 16 known *NF2* exons. We detected 7 mutations in 17 mesotheliomas (41%) within the coding region of *NF2* but none in 75 lung cancer cell lines (38 small cell lung cancers, 34 non-small cell lung cancers, and 3 carcinoids). These mutations were found to be somatic when normal tissue was available for testing. Four mesothelioma cell lines had relatively large deletions (~10–50 kilobases) in the *NF2* gene detectable by Southern blot analysis. Two mesothelioma cell lines had nonsense mutations at codons 57 and 341, respectively. Another mesothelioma obtained as a specimen directly from a patient, had a 10-base pair microdeletion from nucleotide 1004 to nucleotide 1013 causing a frameshift mutation. These results suggest that the *NF2* gene participates in the oncogenesis in a subset of mesotheliomas but not in lung cancers.

Introduction

NF2[3] is an autosomal dominantly inherited disorder characterized by the development of bilateral vestibular schwannomas (acoustic neuromas) of the eighth cranial nerve, frequently accompanied with other disorders such as cranial meningiomas, spinal nerve root schwannomas, and presenile lens opacities (1–3). Cytogenetic and linkage analyses had suggested that the responsible gene for NF2 was localized on chromosome 22q12, leading to the eventual cloning of the target gene (4, 5). The *NF2* gene encodes a 595-amino acid protein, called schwannomin or merlin (for moesin-ezrin-radixin-like protein), that exhibits significant homology to a highly conserved family of proteins that have been postulated to connect the cytoskeleton to components of the plasma membranes. The mutations were detected in multiple tumor types related with the NF2 disorder and also in NF2-unrelated tumors such as melanoma and breast carcinomas (6, 7).

Our published (8, 9) and unpublished cytogenetic studies[4] (see "Materials and Methods" for data) demonstrated that 16 of 28 SCLCs, 17 of 31 NSCLCs, 2 of 3 carcinoids, and 12 of 14 mesotheliomas have abnormalities of chromosome 22 including monosomy 22. Abnormalities suggesting loss of chromosome 22 DNA also have been shown in 16 of 18 SCLCs by the comparative genomic hybridization technique (10), 15 of 32 NSCLCs by molecular analysis of allele loss (11), and mesotheliomas by other cytogenetic studies (12–16). These reports suggest that lung cancer and mesotheliomas may have a mutated tumor suppressor gene such as the *NF2* gene located on 22q. In this study, we surveyed 75 lung cancers and 17 mesotheliomas for *NF2* mutations and detected 7 mesotheliomas that have mutations. However, no mutations were found in lung cancers despite the presence of chromosome 22q cytogenetic abnormalities.

Materials and Methods

Cell Lines and Tumor Samples. The tumor cell lines used in this study have been previously described and some of the lung cancer cell lines and mesothelioma cell lines were deposited at the American Type Culture Collection (Rockville, MD) (17–19). The 75 lung cancer cell lines used in this study included 38 small cell carcinomas, 19 adenocarcinomas, 2 bronchoalveolar carcinomas, 4 squamous cell carcinomas, 2 adenosquamous cell carcinomas, 5 large cell carcinomas, 2 unclassified non-small cell carcinomas, and 3 atypical carcinoids.

Cytogenetic analysis on several NSCLC lines has been reported by Whang-Peng *et al.* (9), while complete cytogenetic analysis on the majority of the lung cancer cell lines was done by H and W Cytogenetic Services, Inc. (Lovettsville, VA). For those cell lines studied cytogenetically, the chromosome 22 findings are given in braces. SCLC cell lines were H82 {no 22 abnormality}, H128 {t(9;22)(pter→q34::q12→qter)}, H146 {no 22 abnormality}, H211 {−22}, H249, H378 {−22}, H524 {−22}, H711, H719, H740 {t(1;22)(p11; q11)}, H748 {t(17;22)(qter→ q11::q11→qter)}, H841 {−22,t(17;22)(qter→ q11::q11→qter)}, H847, H865, H889 {no 22 abnormality}, H1045, H1086 {no 22 abnormality}, H1092 {no 22 abnormality}, H1105 {t(8;22)(qter→q11::q11→qter)}, H1184 {−22}, H1238 {−22,−22}, H1284, H1304 {no 22 abnormality}, H1339 {t(1;22)(qter→p11::q11→qter)}, H1417 {no 22 abnormality}, H1450 {iso(22q)(qter→q11::q11→qter)}, H1514 {no 22 abnormality}, H1618 {no 22 abnormality}, H1628 {−22,−22}, H1672, H1836 {t(15;22)(qter→p11::q11→qter), t(12;22)(qter→q11::p11→qter)}, H1876 {−22}, H1882, H2081 {no 22 abnormality}, H2107 {no 22 abnormality}, H2171 {no 22 abnormality}, H2227 {del(22)(pter→q11)}, and H2258. NSCLC cell lines were H125 {no 22 abnormality}, H157 {−22}, H226 {del(22)(q12)}, H322 {−22}, H358 {no 22 abnormality}, H460 {no 22 abnormality}, H522 {no 22 abnormality}, H647 {−22}, H661 {−22}, H838 {t(17;22)(qter→p11::q11→qter), t(22;?)(qter→p11::?)}, H920 {no 22 abnormality}, H1155 {no 22 abnormality}, H1299 {t(1;22)(qter→q11::p11→pter}, H1334 {t(14;22)(qter→q11::p11→qter)}, H1373 {no 22 abnormality}, H1385 {no 22 abnormality}, H1395 {t(6;22)(q11;q11), t(22;11)(22q;11q; HSR)}, H1437 {iso(22q)(qter→q11::p11→qter)}, H1581 {−22}, H1623 {t(7;22)(p22; q11)}, H1648 {−22}, H1666 {t(11;22)(q13;p11), del(22)(q13)}, H1693 {t(1;22)(pter→q11::q11→qter), t(9;22)(qter→q11::p11→qter), iso(22q)}, H1703, H1755 {no 22 abnormality}, H1770 {no 22 abnormality}, H1781 {no 22 abnormality}, H1792 {no 22 abnormality}, H1869, H1993 {no 22 abnormality}, H2009 {−22}, H2077 {no 22 abnormality}, H2087

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[3] The abbreviations used are: NF2, neurofibromatosis 2; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; SSCP, single-strand conformation polymorphism; RT-PCR, reverse transcriptase PCR; nt, nucleotide.

[4] H. Pass and J. Minna, unpublished results.

{t(9;22)(qter→q11::p11→qter), del(22)(pter→q13)}, and H2286. Carcinoids were H720 {−22}, H727 {−22,−22}, and H835 {no 22 abnormality}.

Cytogenetics on the mesotheliomas were done by the Children's Hospital of Michigan[4] and Dr. J. Whang-Peng (9). The 14 mesothelioma cell lines were H28 {−22}, H290 {del(22)(q11)}, H513 {−22}, H2052 {no 22 abnormality}, H2373 {−22,t(22q;?), t(1q;22q)}, H2452 {−22,−22}, H2461 {−22,−22}, H2591 {−22}, H2595 {no 22 abnormality}, H2596 {−22,del(22)(q12;13)}, H2618 {−22,−22}, HP-1 {−22}, HP-2 {−22}, and HP-3 {−22,del(22)(q11; q13)}. Ten pairs of mesotheliomas (Pt 1 to 10) and normal tissue samples were obtained by Dr. H. Pass during therapeutic operations. Seven of them are original samples from which the corresponding 7 cell lines were established: Pt 1 and HP-1; Pt 2 and H2461; Pt 3 and H2452; Pt 4 and H2373; Pt 5 and HP-3; Pt 9 and H2591; and Pt 10 and H2595.

Primers and PCR/SSCP Analysis. Eight sets of intronic primers for exons 2, 5, 7, 8, 9, 10, 11, and 12 of *NF2* were identical to the previous report for SSCP analysis (7). Another 6 sets of primers (5m1/3m1, 5m2/3m2, 5m3/3m3, 5m4/3m4, 5m5/3m5, and 5m6/3m6) for RT-PCR assay to detect cDNA and to make cDNA probes were identical to those reported (6). To test exon 1 of *NF2*, 2 primers were synthesized: NG ex1(s), 5′-ATGGCCGGGGCCATCGCTT-3′; and NG ex1(as), 5′-CTCGCAATTGAACTCCATCT-3′. To test exon 16 of *NF2*, 2 primers were synthesized: NG31(s), 5′-CCTTCTTTGAAGAGCTC-TAGCA-3′; and NG 32(as), 5′-CCTATGGATGGCTCTCTTGATA-3′.

Each PCR amplification was carried out using 0.5 μg of genomic DNA in a final volume of 20 μl. For SSCP analysis, the reaction was labeled with 0.1 μl (1 μCi) of [α-^{32}P] dCTP (>3000 Ci/mmol; Amersham). PCR conditions consisted of 1 cycle at 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min for most of primers sets. Different annealing temperatures were used for exons 7 and 11 at 60°C and for exons 9 and 10 at 52°C. The labeled PCR products were electrophoretically separated on an MDE gel (J.T. Baker, Phillipsburg, NJ) with or without 10% glycerol at room temperature. For sequencing analysis, aberrant bands were cut from the gel and eluted in 100 μl of H_2O. After PCR products were reamplified using biotinylated primers, single strand DNAs were recovered by streptavidin-coated magnetic beads (Dynabeads, Dynal, Oslo, Norway). Sequencing was performed with a Sequenase version 2 kit (USB Amersham).

Southern and Northern Blot Analyses. For Southern blot analysis, 5 μg of high molecular weight DNA from cell lines were digested by restriction enzymes, electrophoresed, and transferred to Hybond N+ (Amersham). Hybridization and washing conditions were performed by standard techniques (20). For Northern blot analysis, 10 μg of total RNA from cell lines were electrophoresed in formaldehyde-1% agarose gel and transferred to Hybond N (Amersham). Hybridization and washing were performed by standard techniques (20). The DNA probes of PCR-amplified fragments were made by primer sets between 5m1 and 3m1, 5m1 and 3m4, 5m2 and 3m4, and 5m4 and 3m6 for Southern analysis, and between 5m1 and 3m4 for Northern analysis (6).

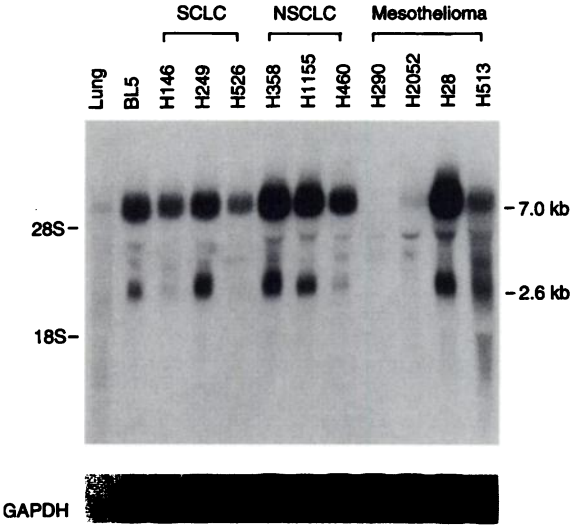

Fig. 1. Northern blot analysis of the *NF2* gene. Each lane was loaded with 10 μg total RNA from normal lung, an Epstein-Barr virus-transformed B cell line (BL5), small cell lung cancer cell lines (H146, H249, and H526), non-small cell lung cancer cell lines (H358, H1155, and H460), and mesothelioma cell lines (H290, H2052, H28, and H513). All cell lines except H290 express 7-kilobase (*kb*) mRNAs of *NF2*. *Bottom*, a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) control for RNA integrity.

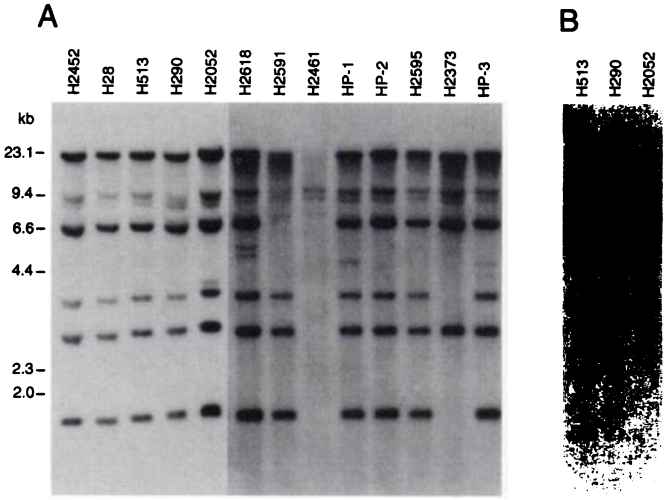

Fig. 2. Deletion of *NF2* in mesotheliomas. *A*, Southern blot analysis of 13 mesothelioma cell lines. DNAs were digested by *Eco*RI and probed with a 1.2-kilobase (*kb*) fragment of RT-PCR products of NF2 amplified by a primer set of 5m1 and 3m4 covering exons 1 to 12 of *NF2*. *B*, Southern blot analysis of 3 mesothelioma cell lines probed with a 330-base pair fragment of *NF2* amplified by a primer set of NG ex1(s) and 3m1 covering exons 1 to 3. The 21-kilobase band corresponds to exons 9–12, the 9.4-kilobase band to exon 1, the 6.6-kilobase band to exons 2–4, the 3.5-kilobase band to exon 7, the 2.8-kilobase band to exons 5 and 6, and the 1.7-kilobase band to exon 8.

Results

Mesothelioma Cell Lines Have Deletions of the *NF2* Gene Detected by Southern Blot Analysis. Expression of NF2 mRNA in lung cancer and mesothelioma was studied using Northern blot analysis and/or RT-PCR analysis. Northern blot analysis showed that the expected strong 7.0-kilobase and weak 2.6-kilobase *NF2* mRNA transcripts were expressed in all lung cancers tested (Fig. 1). However, one of the four mesothelioma cell lines tested, NCI-H290, did not express the 7.0-kilobase mRNA of *NF2* (Fig. 1). Although RT-PCR amplification was performed to detect trace amounts of NF2 mRNA, it also failed to detect these messages in H290 (data not shown).

To test whether the lung cancer and mesothelioma cell lines have gross genomic changes, Southern blot analysis was performed. Genomic DNAs from 75 lung cancer cell lines (38 SCLCs, 34 non-SCLCs, and 3 carcinoids) and 14 mesothelioma cell lines were tested using NF2 cDNA covering exons 1 to 12 amplified by RT-PCR as a probe. Although none of the lung cancer cell lines had Southern blot abnormalities, 4 mesothelioma cell lines exhibited intragenic *NF2* homozygous deletions (Fig. 2). We compared the missing bands with the previously reported restriction map of *Eco*RI digestion (5) and other Southern blots using different parts of the NF2 cDNA amplified by PCR as probes and were able to correlate these missing bands with specific exons of *NF2*. H290 has a deletion of 9.4-kilobase band corresponding to exon 1. This was confirmed by another Southern blot anlysis using cDNA PCR probe covering exons 1 to 3 (Fig. 2*B*). This deletion explains why H290 did not express the 7.0-kilobase NF2 mRNA. H2591 had a deletion of the 6.6-kilobase band corresponding to exons 2 to 4. H2461 had the 9.4-kilobase exon 1 band but not other bands. Another Southern blot using a 3′ NF2 probe showed that H2461 retained an aberrant 8.0-kilobase band which is slightly shorter than the authentic 8.6-kilobase of exon 16 band (data not shown). H2373 had deletions of 3.5- and 1.7-kilobase bands which corresponded to exons 7 and 8, respectively. This cell line also seems to have a

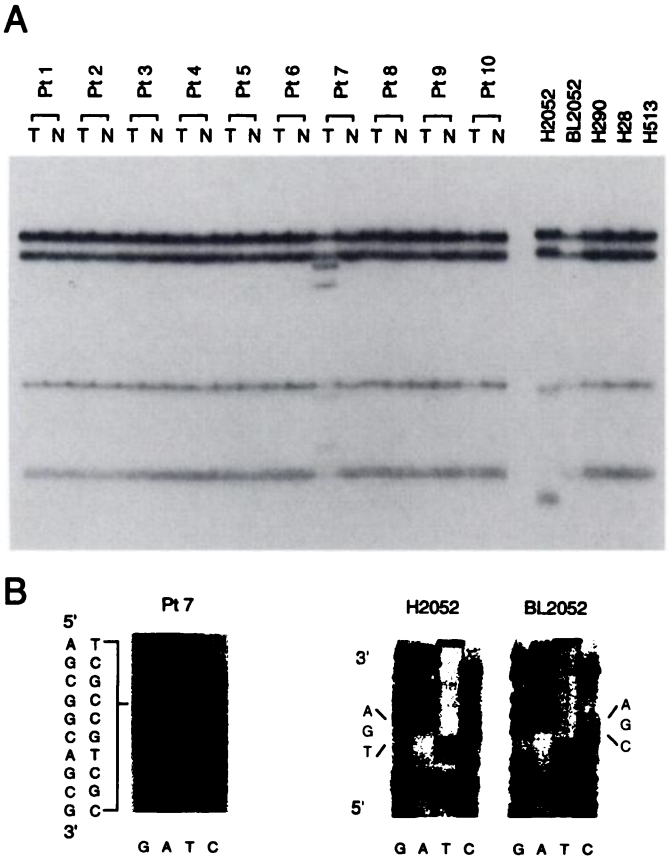

Fig. 3. *A*, identification of distinct electrophoretic mobilities of the *NF2* genomic DNAs in mesotheliomas by PCR-SSCP. Genomic DNAs from tumor (*T*) and normal (*N*) samples from the patients (Pt 1–10) and from 4 mesothelioma cell lines are shown. PCR-SSCP analysis of exon 11 yielded different electrophoretic patterns in the tumor sample from Pt 7 and H2052 but not in their corresponding normal samples. *B*, sequence analysis of the *NF2* genomic DNAs of the mesotheliomas. A 10-base pair microdeletion is observed in the tumor sample from Pt 7. H2052 has a nonsense mutation (TGA) compared to the Epstein-Barr virus-transformed B cell line, BL2052 (CGA, Arg).

relatively smaller band of 21 kilobases corresponding to exons 9 to 15, suggesting that the deletion breakpoint is near exon 9.

To confirm these abnormalities, PCR amplification of 14 mesothelioma cell line DNAs was performed using 10 primer sets to detect exons 1, 2, 5, 7, 8, 9, 10, 11, 12, and 16. These PCR amplifications did not detect any products for exon 1 of H290; for exon 2 of H2591; for exons 2, 5, 7, 8, 9, 10, 11, and 12 of H2461; and for exons 7 and 8 of H2373 (data not shown). Thus, the data of PCR amplification completely matched with the Southern blot analysis. We also had a sample of fresh tumor DNA from which mesothelioma cell lines H2591 (Pt 9), H2461 (Pt 2), and H2373 (Pt 4) were derived. PCR analysis of the fresh tumor samples gave a PCR signal consistent with the presence of normal cells admixed with tumor cells, although all of these lines had *NF2* deletions.

Searching for Subtle Mutations in the *NF2* Gene. To test whether the *NF2* gene in lung cancer and mesothelioma had point mutations or small deletions, we performed SSCP analysis. Since 8 of the known 16 exons of *NF2* had been tested in sporadic meningiomas by Ruttledge *et al.* (7), resulting in detection of many mutations, we synthesized identical primer sets for our analysis. We tested genomic DNAs from 75 lung cancer cell lines and 14 mesothelioma cell lines described above and 10 mesothelioma samples from patients (7 of these were from the same patients giving rise to 7 of the mesothelioma cell lines). SSCP analysis showed that DNA from 2 mesotheliomas produced patterns different from that of the wild type in exon 11 (Fig. 3*A*) and 1 mesothelioma was different in exon 2 (data not shown). These bands were cut from gels, reamplified using biotinylated primers, and sequenced. The mesothelioma cell line, NCI-H2052, had a T to C change at nt 1021 resulting in a nonsense mutation (codon 341, Arg to Stop) (Fig. 3*B*). This was confirmed to be a somatic mutation by comparing tumor DNA to the corresponding immortalized B lymphocyte cell line established from the same patient. The other mesothelioma sample from patient 7, whose normal sample did not have such an aberrant band thus indicating that this change was also a somatic mutation, was shown to have a 10-base pair microdeletion from nt 1003 to nt 1012 resulting in a frame-shift mutation (Fig. 3*B*). Another cell line, HP-3, had a C to T change at nt 169 causing a nonsense mutation at codon 57 (Arg to Stop) (data not shown). Fig. 4 summarizes the 7 *NF2* abnormalities found in the mesotheliomas.

Discussion

The data presented here are the first systematic examination of the *NF2* gene for mutation in thoracic malignancies. Southern blot analysis detected 4 genomic changes among 14 mesothelioma cell lines

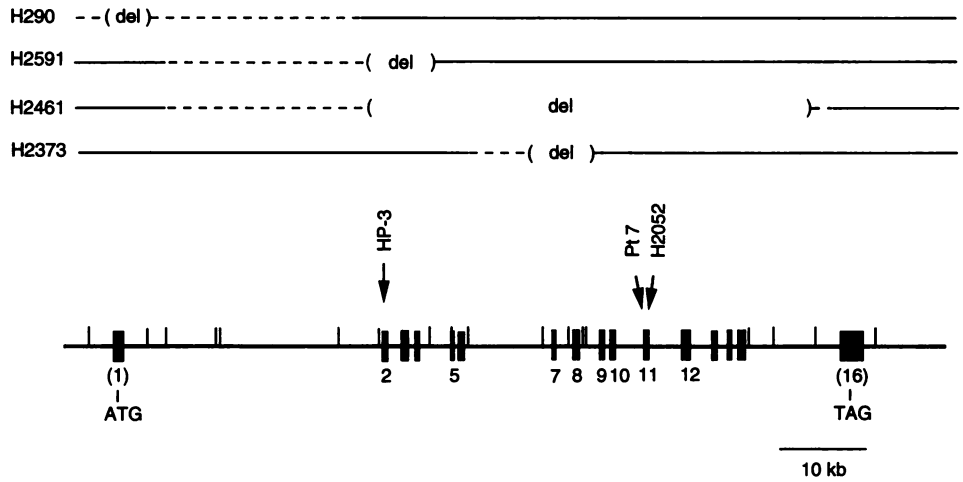

Fig. 4. Schematic representation of the abnormalities of the *NF2* gene in mesotheliomas from 6 cell lines and 1 tumor sample (Pt 7). The genomic map of *NF2*, the locations of 16 exons, and *Eco*RI sites were reported by Rouleau *et al.* (5). Four mesotheliomas have deletions of *NF2* and 3 mesotheliomas have point mutations or a microdeletion. (del), deletion; ——, retained regions; – – – –, undetermined regions (as determined by Southern blot and PCR analyses). ■, exons. *Numbers* under exons were analyzed by SSCP analysis. Exons 1 and 16 with *parentheses* indicate that PCR analyses were performed to detect retention of each exon. *Vertical lines*, *Eco*RI sites. *kb*, kilobase.

but none in 75 lung cancer cell lines. Our SSCP analysis covered 937 base pairs of the 1785-base pair (53%) coding nucleotides of the *NF2* cDNA. SSCP analysis demonstrated that 3 other mesotheliomas have *NF2* mutations and 2 were confirmed to be somatic mutations. In total, 7 of 17 (41%) individual mesotheliomas have genetic changes of *NF2*, while in contrast no *NF2* mutations were found in 75 lung cancer cell lines. Interestingly, all seven alterations are predicted to generate truncated or no proteins as the result of in-frame deletions or a premature stop codon. This finding is compatible with the previous evidence that almost all mutations observed in sporadic meningiomas, sporadic vestibular schwannomas, melanomas, and a breast cancer were either in-frame deletions, splice-site alterations, or nonsense mutations resulting in a truncated and presumably inactive protein (6, 7).

Fletjer *et al.* (15) reviewed data showing that clonal abnormalities of chromosome 22 in mesothelioma had been observed in 16 of 28 cases (57%). Abnormalities of chromosome 22 were characterized by monosomy (11 of 16), partial nullisomy (1 of 16), and other structural rearrangements (4 of 16). Overall, monosomy 22 was scored to be the single most consistent numerical change seen in malignant mesothelioma. Our cytogenetic analysis of the mesothelioma cell lines also detected frequent chromosome 22q abnormalities (9)[4] (see "Materials and Methods"). Monosomy 22 was observed in H28, H513, H2591, HP-1, and HP-2. No intact chromosome 22 was seen by standard banding cytogenetic analysis in H2452, H2461, and H2618. Cell lines H2373, H2596, and HP-3 also show chromosome 22 fragments as part of marker chromosomes. The frequency of *NF2* point mutations in mesothelioma may increase if all of the exons were covered since only eight exons (2, 5, 7, 8, 9, 10, 11, and 12) were studied. Therefore, the frequency of mutated cases in this study (7 of 17, 41%) may account for a significant part of the 22q abnormalities in mesotheliomas detected by cytogenetic data. We have also found chromosome 22q cytogenetic abnormalities in many lung cancer cell lines (9),[4] and molecular analysis has indicated 22q allele loss in NSCLC (11). The results of this study show a lack of mutation of the *NF2* gene in lung cancers despite these chromosome 22q changes and suggest the presence of an additional tumor suppressor gene on chromosome 22q.

Abnormalities were found in 6 of 14 mesothelioma cell lines and 1 of 10 tumor samples. Three of the cell lines derived from these tumor samples had a deletion not detected by PCR analysis in the tumor, presumably because of a mixture of tumor and normal cells in the fresh sample. However, we cannot exclude the possibility that these deletions occurred during cell culture.

Malignant mesothelioma is a very aggressive disease clinically with uniformly poor response to current therapeutic regimens. Although several chromosomes have been reported to harbor frequent abnormalities, there are very few reports to demonstrate which tumor suppressor gene is responsible for this malignancy. For example, *p53* was demonstrated to be mutated in 67% (2 of 3) of mesotheliomas (21), and 70% (14 of 20) of malignant mesotheliomas showed *p53* immunoreactivity (22). On the other hand, SV40-like DNA sequences were detected in 29 of 48 mesotheliomas, and SV40 large T antigen expression was found in 13 of 16 mesothelioma specimens (23). These findings are of considerable interest since large T antigen can inactivate tumor suppressor proteins such as p53 and RB (24) and would suggest a lack of requirement for mutation of *p53*. Therefore, the role of viral related T antigen in mesotheliomas will require future study. A mutation on another tumor suppressor gene, *WT1*, was reported in a multicystic peritoneal mesothelioma arising within the omentum of a 45-year-old woman but not in 32 specimens of asbestos-related mesothelioma (25). Another report showed that all of seven mesothelioma cell lines expressed *WT1* transcripts, suggesting that *WT1* expression might be involved in the tumorigenesis of mesothelioma and could be useful for diagnosis (26). Our report provides the first definitive evidence suggesting that a third tumor suppressor gene, *NF2*, is mutated in malignant mesothelioma. Although the function of NF2 is still unclear, Tikoo *et al.* (27) reported that transfection of *NF2* suppressed the malignant phenotype of v-Ha-*ras* transformed NIH/3T3 cells. It suggests that an antitumor function of *NF2* is involved in the *ras* signal transduction pathway.

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