

A Mouse Model Recapitulating Molecular Features of Human Mesothelioma

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Abstract

Malignant mesothelioma has been linked to asbestos exposure and generally has a poor prognosis because it is often diagnosed in advanced stages and is refractory to conventional therapy. Human malignant mesotheliomas accumulate multiple somatic genetic alterations, including inactivation of the *NF2* and *CDKN2A/ARF* tumor suppressor genes. To better understand the significance of *NF2* inactivation in malignant mesothelioma and identify tumor suppressor gene alterations that cooperate with *NF2* loss of function in malignant mesothelioma pathogenesis, we treated *Nf2* (+/−) knockout mice with asbestos to induce malignant mesotheliomas. Asbestos-exposed *Nf2* (+/−) mice exhibited markedly accelerated malignant mesothelioma tumor formation compared with asbestos-treated wild-type (WT) littermates. Loss of the WT *Nf2* allele, leading to biallelic inactivation, was observed in all nine asbestos-induced malignant mesotheliomas from *Nf2* (+/−) mice and in 50% of malignant mesotheliomas from asbestos-exposed WT mice. For a detailed comparison with the murine model, DNA analyses were also done on a series of human malignant mesothelioma samples. Remarkably, similar to human malignant mesotheliomas, tumors from *Nf2* (+/−) mice showed frequent homologous deletions of the *Cdkn2a/Arf* locus and adjacent *Cdkn2b* tumor suppressor gene, as well as reciprocal inactivation of *Tp53* in a subset of tumors that retained the *Arf* locus. As in the human disease counterpart, malignant mesotheliomas from the *Nf2* (+/−) mice also showed frequent activation of Akt kinase, which plays a central role in tumorigenesis and therapeutic resistance. Thus, this murine model of environmental carcinogenesis faithfully recapitulates many of the molecular features of human malignant mesothelioma and has significant implications for the further characterization of malignant mesothelioma pathogenesis and preclinical testing of novel therapeutic modalities. (Cancer Res 2005; 65(18): 8090-5)

Introduction

Exposure to asbestos fibers is the primary cause of malignant mesothelioma (1), with 80% of malignant mesotheliomas in the Western World developing in individuals with higher than background exposure to asbestos. However, <10% of people heavily

exposed to asbestos develop malignant mesothelioma, suggesting that additional carcinogens or predisposing genetic factors are also involved in the etiology of this disease. A multistep genetic progression has been suggested in malignant mesothelioma (2) because several prominent sites of chromosomal loss have been identified, with a combination of recurrent abnormalities usually present in a given tumor (3). Human malignant mesotheliomas often exhibit inactivation of tumor suppressor genes such as *NF2* (4, 5), and we have previously shown that p21-activated kinase is activated as a result of *NF2* inactivation in malignant mesothelioma cells (6). In addition, homozygous deletion of the *CDKN2A/ARF* locus, encoding the tumor suppressors p16(INK4a) and p14(ARF), is often observed in malignant mesotheliomas (7), whereas *TP53* mutations are seen less frequently (8). Activation of the serine-threonine kinase AKT/protein kinase B, which is thought to play a central role in tumorigenesis, is also common in human malignant mesothelioma (9). AKT activation may cooperate with alterations of specific tumor suppressor genes in malignant mesothelioma pathogenesis, and recent findings suggest AKT activation may be predictive of chemotherapeutic resistance. To date, it has not been established that molecular alterations characteristic of human malignant mesothelioma are recapitulated in a rodent model of the disease. Indeed, inactivation of *Nf2* was not observed in asbestos-induced rat malignant mesotheliomas (10). Here we report a mouse model of asbestos-induced carcinogenesis that develops malignant mesotheliomas with molecular alterations, considered to be hallmarks of human mesothelial cell tumorigenesis, including inactivation of key tumor suppressor genes and activation of Akt. These findings suggest that this mouse model may be invaluable for the further characterization of malignant mesothelioma pathogenesis and for the design of novel therapies and, potentially, preventive measures sorely needed to combat this uniformly fatal form of cancer.

Materials and Methods

Animals. Mice were housed and treated according to guidelines established by the NIH Guide for the Care and Use of Laboratory Animals. Heterozygous *Nf2* (+/−) mice, designed to mimic human *NF2* syndrome, develop mainly osteosarcomas with a mean latency of ~20 months (11). Male *Nf2* (+/−) and wild-type (WT) littermate mice were derived from crosses between *Nf2* (+/−) mice in an inbred 129Sv/Jae background. Tail DNA was genotyped as described (11).

Fibers and treatment of mice. Unio Internationale Contra Cancrum (UICC)-grade crocidolite asbestos was obtained from SPI Supplies (West Chester, PA), and titanium dioxide particles (TiO₂) were obtained from Aldrich Chemicals (Milwaukee, WI). As a pilot experiment, the total numbers of cells and neutrophils in peritoneal lavage from mice injected i.p. with crocidolite (100, 200 and 500 μg doses) were assessed at 3, 7, and 14 days after treatment. Toxicity was comparable to another lot of UICC-grade

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crocidolite used in a previous study of *Tp53*-deficient mice (12), although crocidolite used here had a higher percentage of long fibers.

We modified the methods used to induce malignant mesothelioma in *Tp53*-deficient mice (12) and repeatedly injected *Nf2* (+/−) mice with crocidolite asbestos or control TiO₂ particles. Mice, 6 to 8 weeks of age, were injected i.p. with 400 μg of crocidolite in 1 mL of PBS every 21 days for a total of eight rounds of injections (total: 3.2 mg crocidolite per mouse). A corresponding dose of 320 μg of TiO₂, containing approximately the same number of particles as asbestos fibers, was administered using the same regimen. A total of 14 *Nf2* (+/−) mice and 15 WT littermates were treated with TiO₂, and 35 *Nf2* (+/−) and 32 WT mice were treated with asbestos, although 1 *Nf2* (+/−) and 5 WT mice from the asbestos-treated groups were not analyzable for cause of death. Bedding, cages, and euthanized mice were incinerated to dispose of potentially contaminated material. Complete necropsies were done on all mice, and histopathologic diagnosis of malignant mesotheliomas was as previously described (13).

Primary cell cultures. Primary murine malignant mesothelioma cells were isolated from ascitic fluid and/or lavage of the peritoneal cavity using PBS. Cells were washed in PBS and placed in DMEM with 20% FCS, supplemented with 2 mmol/L L-glutamine and 10 units/mL penicillin/streptomycin. Cells from passage 2 to 9 were used for molecular analyses.

PCR and reverse transcription-PCR. DNA and total RNA from tails or cultured murine malignant mesothelioma cells were isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Genomic DNA from human malignant mesothelioma cells was isolated as previously described (7). Primers for mouse WT and knockout *Nf2*, as well as primers for mouse or human *p16(INK4a)*, *p19(Arf)*, *p15(Ink4b)*, and WT *Tp53*, were designed based on previous investigations (7, 11) or on National Center of Biotechnology Information (NCBI)⁶ database information. Additional reverse transcription-PCR (RT-PCR) primers for WT1, cytokeratin 18, cytokeratin 19, E-cadherin, and N-cadherin for expression analyses were also designed based on NCBI database information. Primers for murine β-actin were purchased from Clontech Laboratories (Palo Alto, CA).

Western blotting. Western blot analysis of protein lysates was done as previously described (9). Actin expression was used as a control to assess protein extract quality. Protein, 15 to 30 μg, was subjected to Western blot analysis. Primary antibodies included NF2 A-19 and p53 FL-393 (Santa Cruz Biotechnology, Santa Cruz, CA). Detection of antigen-bound antibody was carried out with the Renaissance Chemiluminescence Reagent Plus system (NEN Life Science, Boston, MA).

***Tp53* sequencing.** Genomic DNA was isolated from primary murine malignant mesothelioma cell cultures, and exons 4 to 11 of the *Tp53* gene were PCR amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA), as previously described (14). The products were cloned into a pCRII-Topo vector (Invitrogen) and sequenced using *Tp53* intron-specific forward and reverse primers. Human *TP53* exons 4 to 11 were PCR amplified using primers designed with NCBI database information.

Results

Following repeated injections of crocidolite asbestos, we found markedly accelerated malignant mesothelioma tumor formation in asbestos-treated *Nf2* (+/−) mice compared with asbestos-treated WT littermates (Fig. 1A and B), whereas none of the control TiO₂-treated mice developed malignant mesothelioma. A log-rank test showed that *Nf2* (+/−) mice had markedly decreased survival times compared with WT mice ($P = 1.54 \times 10^{-9}$ for the inclusion of all mice; $P = 5.58 \times 10^{-7}$ for the subset of mice that that developed malignant mesothelioma).

The prolonged latent period, pattern of tumor growth, and histopathologic appearance of malignant mesothelioma in WT mice resembled those described in previous rodent models (15). Fibrous

adhesions and ascites (Fig. 2A) developed earlier in *Nf2* (+/−) mice, and the rate of tumor dissemination and invasion was also accelerated in *Nf2* (+/−) mice. As illustrated in Fig. 2, malignant mesothelioma initially spread diffusely over the serosal surfaces and then grew as detached masses or spheroids suspended in serosanguinous ascitic fluid. These spheroids then implanted on the visceral and parietal mesothelial lining; invasive tumors frequently infiltrated the skeletal muscle of the diaphragm or mesenteric fat. Metastases were usually limited to the peritoneum and were characterized by invasion of subserosal lymphatics.

Because Akt regulates cellular processes that contribute to important hallmarks of cancer, including cell survival, growth and proliferation, glucose metabolism, genome stability, and neo-vascularization, and we previously reported that human malignant mesotheliomas and malignant mesotheliomas from WT mice often exhibit elevated Akt activity (9), we did immunohistochemical analyses with a Ser⁴⁷³ phospho-specific pan-Akt antibody, which recognizes the active form of Akt kinases, on nine tumors from asbestos-treated *Nf2* (+/−) mice. All tissue sections exhibited strong phospho-Akt staining in malignant mesothelioma cells compared with surrounding stroma (Fig. 2F). Cells isolated from ascites or peritoneal lavage were cultured, and RT-PCR confirmed the expression of mesothelial-specific histologic markers. Overall, all nine tested cultures from *Nf2* (+/−) mice expressed cytokeratins 18 and 19 and N-cadherin; 8 of 9 (89%) cultures expressed WT1, and 6 of 9 (67%) expressed E-cadherin (data not shown).

As predicted, we found consistent biallelic inactivation of *Nf2* in malignant mesotheliomas from *Nf2* (+/−) mice, with loss of the WT *Nf2* allele observed in all nine primary malignant mesothelioma cultures analyzed, whereas retention of the targeted knockout fragment was evident in all but one tumor (Fig. 3A). RT-PCR analysis of *Nf2* transcripts and Western blot analysis of tumor cell lysates confirmed the DNA results (Fig. 3A and B). Of note, biallelic inactivation of *Nf2* was also observed in 4 of 8 (50%) malignant mesotheliomas from asbestos-treated WT mice (Fig. 3B), and this lower frequency of biallelic *Nf2* inactivation is similar to the incidence of biallelic inactivation of *NF2* that we have documented in human malignant mesotheliomas (5).

Our previous deletion mapping analyses of human malignant mesothelioma cell lines uncovered frequent homozygous deletions of chromosome 9p21 (16), which contains the tumor suppressor genes *p16(INK4A)*, *p14(ARF)*, and *p15(INK4B)*. We found that malignant mesothelioma cells from asbestos-treated *Nf2* (+/−) mice frequently exhibited homozygous deletions of these same tumor suppressor genes. Genomic and RT-PCR analyses revealed codeletions of *p16(INK4A)* and *p19(Arf)* in 6 of 9 (67%) malignant mesotheliomas from *Nf2* (+/−) mice (Fig. 3A). Homozygous deletions of *p15(INK4B)* only occurred in conjunction with homozygous loss of *p16(INK4A)* and *p19(Arf)*. In the *Nf2* (+/−) mice, loss of *Tp53* was detected by RT-PCR and loss of p53 protein was confirmed by immunoblotting in 4 of 9 (44%) malignant mesotheliomas. No deletions or missense mutations were found in the malignant mesothelioma cell cultures that retained WT *Tp53*. Interestingly, we observed that expression of *Tp53* was lost in primary murine malignant mesothelioma cell cultures that retained *p19(Arf)*, and vice versa (Fig. 3A).

We had previously identified homozygous deletions of *p16(INK4A)* in 34 of 40 (85%) human malignant mesothelioma cell lines tested (7). We have subsequently conducted further deletion mapping studies, which revealed homozygous deletions of part or all of *p14(ARF)* in 36 of the 40 (90%) human malignant

⁶ <http://www.ncbi.nlm.nih.gov>.

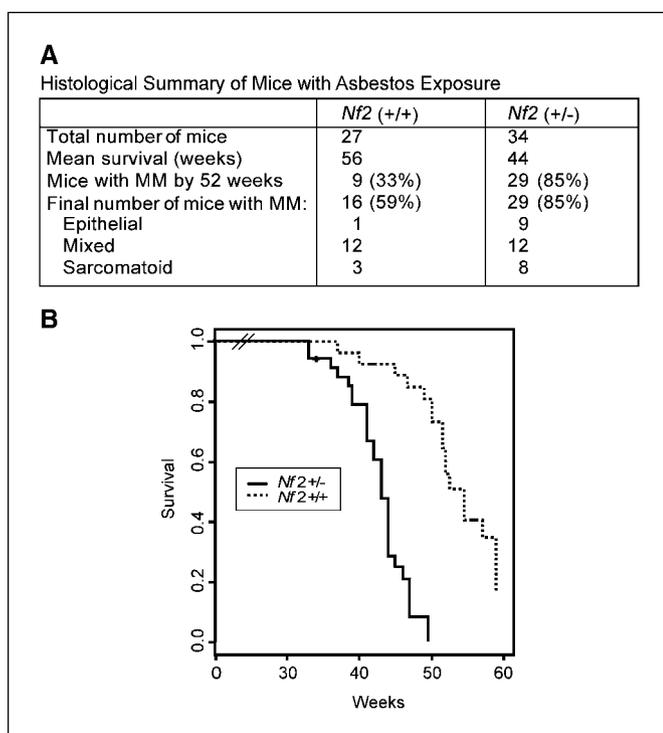


Figure 1. Asbestos-treated *Nf2* (+/-) mice exhibit decreased malignant mesothelioma tumor latency compared with asbestos-treated WT littermates. **A**, summary of malignant mesothelioma tumor development, survival, and tumor subtype in mice exposed to asbestos. **B**, comparison of survival in asbestos-treated *Nf2* (+/-) and WT mice, depicted by Kaplan-Meier survival curves. *Nf2* (+/-) mice were found to have significantly shorter survival times than WT mice using the log-rank test, and a statistically significant difference was found between the two groups ($P = 1.54 \times 10^{-9}$). Sixteen mice had no tumors or had incidental tumors and were excluded as censored observations. Using Fisher's exact test, the association between genotype, *Nf2* (+/-) or *Nf2* (+/+), and incidence of malignant mesothelioma was tested. At the 5% significance level, there is a statistically significant association between genotype and incidence of malignant mesothelioma (two-sided $P = 0.039$). The odds of malignant mesothelioma incidence are four times as high in the *Nf2* +/- mice as in the WT mice.

mesothelioma cell lines, with codeletion of *p16(INK4A)* and *p14(ARF)* in most malignant mesotheliomas (Fig. 4A). Among these 36 cases, 7 had partial deletions of *p14(ARF)*, including 3 with homozygous deletions of exon 1 β only. Homozygous deletions of *p15(INK4B)* occurred less frequently (32 cell lines) and, as in the murine malignant mesotheliomas, never occurred in the absence of a homozygous loss in the *INK4a/ARF* locus. Because *p14(ARF)* and *p53* are functionally linked, we decided to evaluate a subset of our human malignant mesotheliomas to determine if, as was the case in the murine malignant mesotheliomas, there is reciprocity between *TP53* and *p14^{ARF}* alterations. We did an assessment of the mutational status of *TP53* in 20 malignant mesothelioma cell lines and matched tumor specimens. Analysis of *TP53* exons 4 to 11 revealed mutations in 3 of 20 (15%) malignant mesothelioma cell lines tested. The same mutations were observed in the corresponding tumor samples, whereas WT *TP53* was present in matched normal cells. Interestingly, two tumor samples with *TP53* mutations did not show homozygous loss of *p14(ARF)*, indicating that perturbation of the *p53* pathway in a given malignant mesothelioma can arise due to defects in either *p14(ARF)* or *p53*. Altogether, 19 of 20 malignant mesothelioma cases had an alteration affecting the *p14(ARF)/p53* pathway (Fig. 4B), providing further support for a critical role of this pathway in the pathogenesis of malignant mesothelioma.

Discussion

Germ line mutations of *NF2* predispose individuals to tumors of neuroectodermal origin, but somatic mutations of *NF2* can also occur in sporadic meningiomas and schwannomas and, occasionally, in unrelated malignancies. We previously detected somatic truncating mutations of *NF2* in ~50% of our malignant mesotheliomas, and similar results have been reported by others (4, 5). Of note, we recently reported an asbestos-exposed patient with *NF2* syndrome and malignant mesothelioma, and we proposed that an individual with a germ line mutation of *NF2*

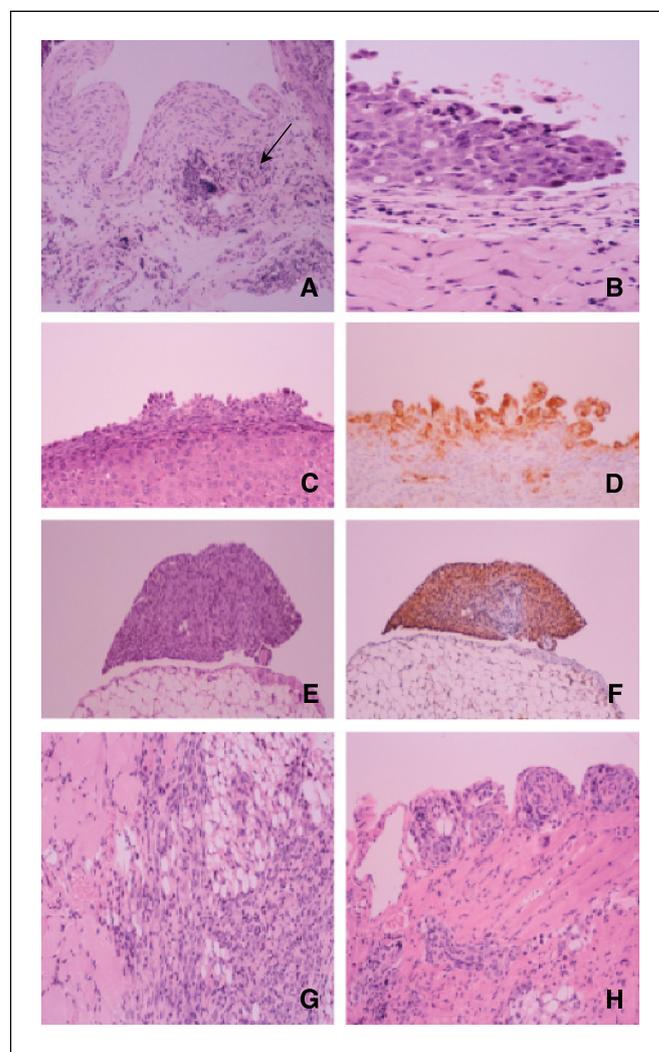


Figure 2. Histopathology of *Nf2* (+/-) mice following repeated i.p. injections of crocidolite asbestos fibers. **A**, fibrous mesenteric adhesions surrounding clusters of asbestos fibers within macrophages and multinucleated giant cells (arrow) at $\times 25$ magnification stained with H&E. **B**, localized malignant mesothelioma on the surface of the diaphragm at $\times 100$ magnification stained with H&E. Tumor cells are exfoliating from the surface. **C**, diffuse epithelial malignant mesothelioma on the surface of the liver at $\times 50$ magnification stained with H&E. **D**, cytoplasmic expression of high-molecular weight cytokeratins by epithelial malignant mesothelioma cells illustrated in **C** at $\times 50$ magnification; 3,3'-diaminobenzidine (DAB) immunohistochemistry with hematoxylin counterstain. **E**, tumor spheroid at $\times 25$ magnification stained with H&E. **F**, cytoplasmic expression of phospho-AKT in the spheroid illustrated in **E** at $\times 25$ magnification; DAB immunohistochemistry. **G**, sarcomatoid malignant mesothelioma invading into skeletal muscle and mesenteric fat at $\times 50$ magnification stained with H&E. **H**, lymphatic invasion at $\times 50$ magnification stained with H&E.

may have increased susceptibility to malignant mesothelioma (17). However, the clinicopathologic consequences of *Nf2* alterations of malignant mesothelioma are not well defined. In a previous study utilizing *Nf2* (KO3/+) mice with insertional inactivation of *Nf2* exon 3, only one spontaneous malignant mesothelioma was found in a cohort of 16 *Nf2* (KO3/+) mice, although these mice exhibited increased susceptibility to asbestos-induced malignant mesothelioma (18). In the mouse model presented here, heterozygous *Nf2* mice not only showed increased susceptibility to asbestos-induced malignant mesothelioma but also consistently exhibited a profile of molecular perturbations, including specific tumor suppressor gene alterations and activation of Akt, which recapitulate features of human malignant mesothelioma.

Overall, a variety of epithelial, sacromatoid, and mixed histologic subtypes of malignant mesotheliomas expressing mesothelial-specific markers were observed in both *Nf2* (+/-) and WT mice. Moreover, strong Akt activation in the malignant mesotheliomas arising in asbestos-induced *Nf2* (+/-) mice is consistent with our

previous findings in human malignant mesotheliomas and malignant mesotheliomas from WT mice treated with asbestos (9), suggesting that the *Nf2* (+/-) mouse model may be useful for elucidating the role of AKT activation in malignant mesothelioma cell survival and drug resistance.

Importantly, our analyses of malignant mesotheliomas from asbestos-treated *Nf2* (+/-) mice and expanded studies of human malignant mesotheliomas revealed markedly similar somatic genetic changes, including homozygous deletion of the tumor suppressor genes *p16*(*INK4A*), *p14*(*ARF*)/*p19*(*Arf*), and/or *p15*(*INK4B*). Moreover, in both mouse and human malignant mesotheliomas, a similar reciprocal pattern of ARF loss versus p53 alteration was observed. Whereas a detailed analysis of the malignant mesothelioma cell lines derived from asbestos-treated WT mice was not done, Western blot analysis showed loss of the Nf2 protein, merlin, in 50% of asbestos-induced malignant mesotheliomas, which is lower than the incidence found in malignant mesotheliomas from *Nf2* (+/-) mice but similar to the

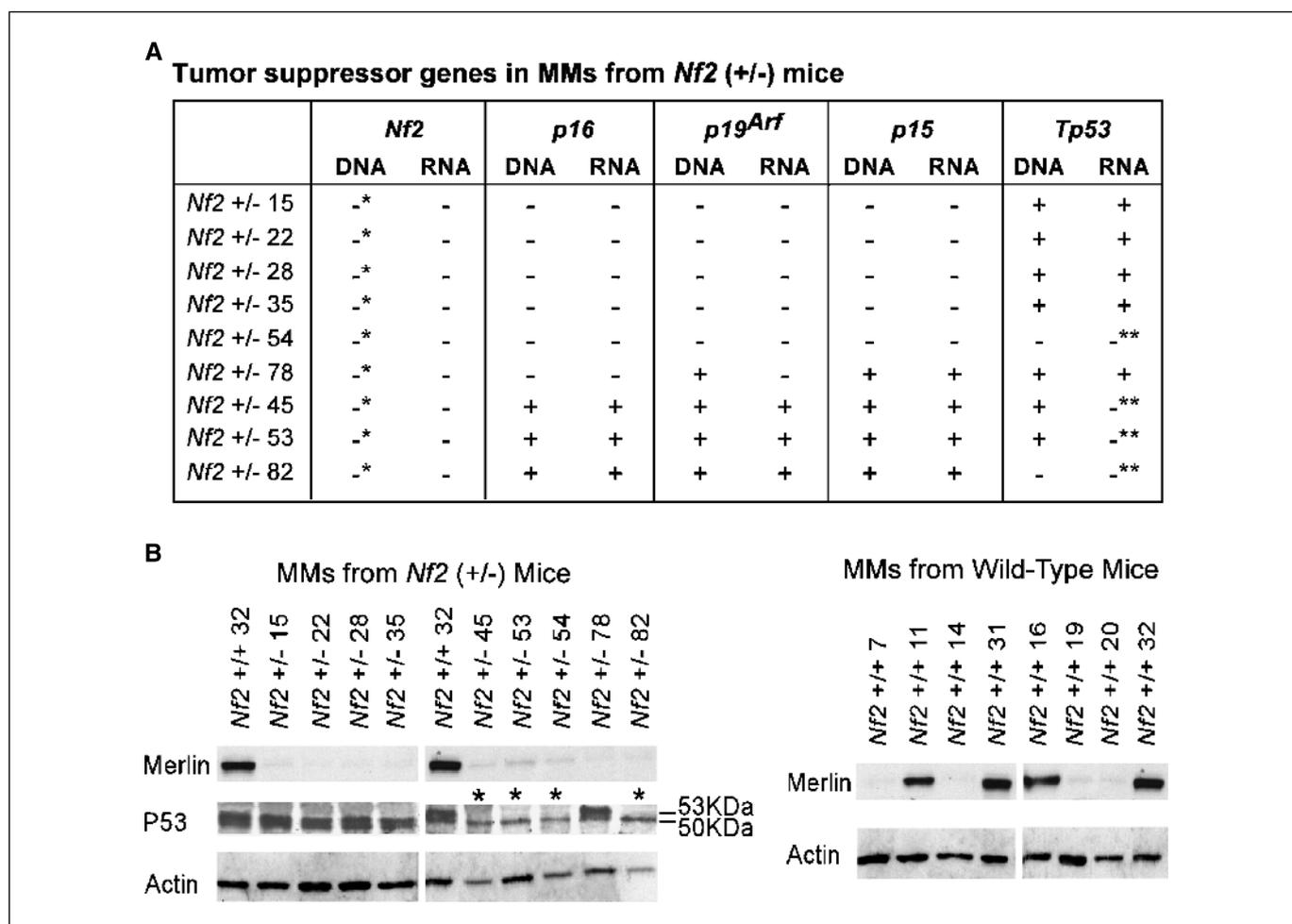


Figure 3. Molecular alterations in malignant mesotheliomas from *Nf2* (+/-) mice recapitulate those of human malignant mesotheliomas, including biallelic inactivation of *Nf2*, homologous loss of *p16*(*Ink4a*), *p19*(*Arf*), and *p15*(*Ink4b*), loss of *Tp53*, and activation of Akt. **A**, summary of PCR assays for tumor suppressor genes in malignant mesotheliomas from *Nf2* (+/-) mice. Note the loss of the WT *Nf2* allele in all *Nf2* (+/-) mice (asterisk). Double asterisks, loss of p53 protein on Western blots. Note the reciprocal loss of either *p19*(*Arf*) or *Tp53* in individual malignant mesothelioma cultures [e.g., loss of p53 expression in malignant mesothelioma cultures 45, 53 and 82, each of which retained *p19*(*Arf*)]. **B**, Western blots of protein lysates from malignant mesothelioma cells (passage 2) from *Nf2* (+/-) mice and WT littermates. The *Nf2* product, merlin, is absent in all nine malignant mesotheliomas examined from *Nf2* (+/-) mice, and also absent in four of eight malignant mesotheliomas from WT mice. Loss of p53 expression was observed in four of nine malignant mesotheliomas from *Nf2* (+/-) mice. Note that the p53 antibody detects a smaller 50 kDa background band, but the 53 kDa band corresponding to p53 was not detectable in malignant mesothelioma cell lines that lacked the *p53* transcript (asterisk).

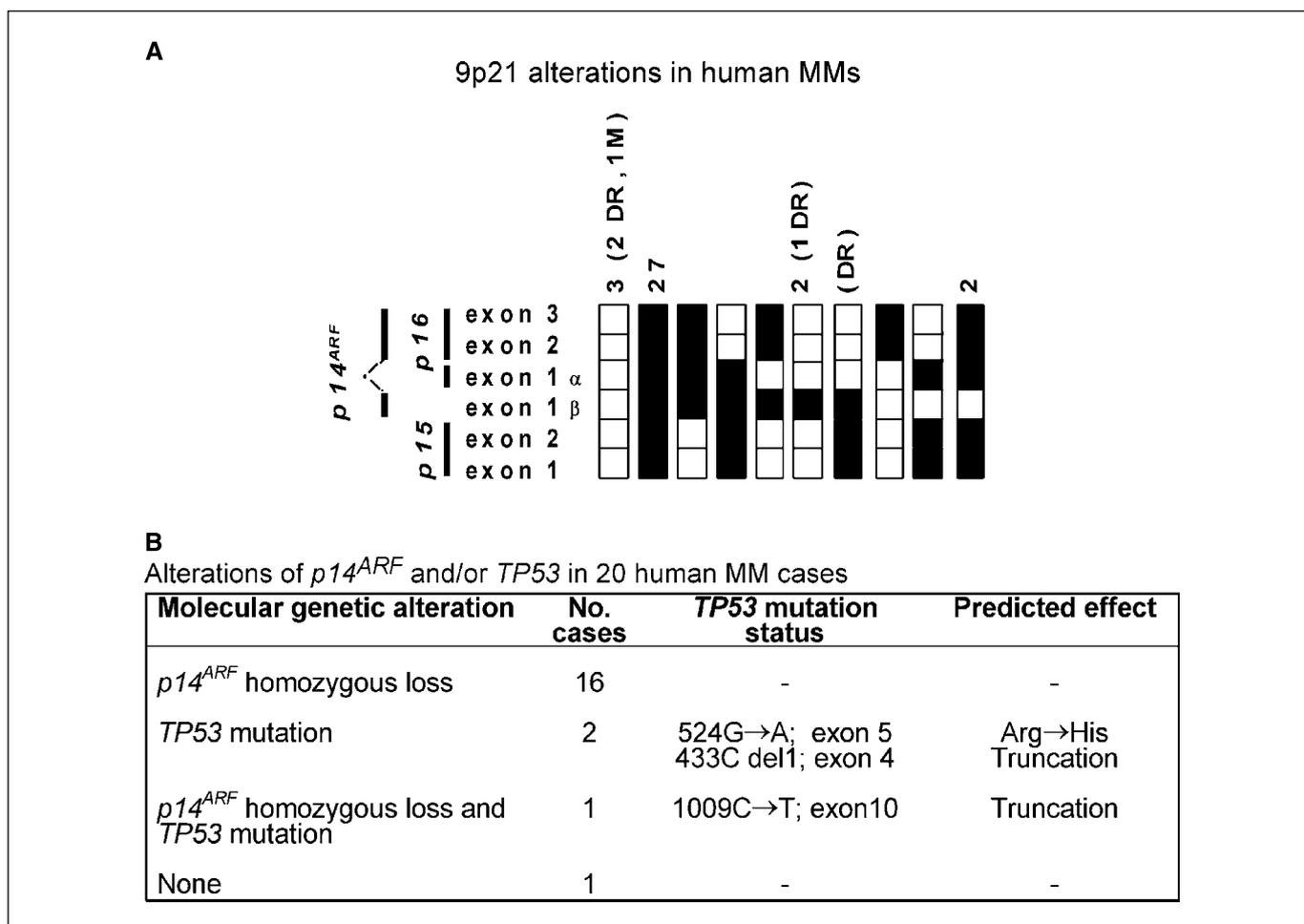


Figure 4. Summary of tumor suppressor gene alterations in human malignant mesothelioma cell lines. **A**, deletion mapping revealed homozygous deletions of part or all of *p14^{ARF}* in 36 of the 40 (90%) malignant mesothelioma cell lines described above, with codeletion of *p16(INK4A)* and *p14(ARF)* in most malignant mesotheliomas. Orientation of *p16(INK4A)*, *p14(ARF)*, and *p15(INK4B)* exons is depicted from telomere (upper) to centromere (lower). *Black box*, homozygous loss; *open box*, retention of individual exon. *Numbers on top*, particular loss profiles seen in multiple malignant mesotheliomas. *DR*, down-regulation of *p16(INK4A)* in the absence of a homozygous deletion. *M*, case with point mutation in *p16(INK4A)*. The extent of individual homozygous deletions in 9p21 beyond the depicted region varied among cases. **B**, summary showing the association between molecular alterations of *p14(ARF)* and *TP53* in 20 human malignant mesothelioma cases.

frequency of *NF2* inactivation reported in human malignant mesotheliomas (5). Taken together, these data implicate a common set of cellular perturbations in both human and mouse malignant mesotheliomas. Thus, alterations of the p53/ARF and p16(INK4A) cell cycle regulatory pathways and the AKT and p21-activated kinase-merlin signal transduction pathways seem to be critical events that cooperate to drive malignant mesothelioma tumorigenesis in both human and murine malignant mesotheliomas. These findings are consistent with cancer being a multistep process involving the accumulation of somatic genetic changes that enable tumor cells to override fail-safe mechanisms regulating normal cell proliferation (19).

The studies presented here establish a framework for further elucidating the pathogenesis of malignant mesothelioma in a mouse model of environmental carcinogenesis. Based on the collective evidence presented here, our working hypothesis is that alteration of either p14(ARF) or p53 would permit malignant mesothelioma cells to circumvent an essential cell cycle checkpoint and continue to divide despite sustaining asbestos-induced DNA damage; loss of p16(INK4a) and/or up-regulation of cyclin D1,

the latter resulting from loss of NF2 expression (6), would deregulate the retinoblastoma pathway and promote cell cycle progression. Activation of the AKT pathway would be expected to play a role in overcoming other fail-safe mechanisms regulating normal cell proliferation by promoting cell survival and protein translation required to maintain a high rate of cell growth (20). p21-activated kinase signaling has been shown to promote cell motility (21), and expression of exogenous merlin inhibits p21-activated kinase activity in NF2-deficient malignant mesothelioma cells (6), suggesting that NF2 loss could contribute to tumor cell migration and/or invasiveness. Furthermore, older *Nf2*-deficient mice have been shown to develop a wide range of highly metastatic tumors (11). The potential role of *Nf2* in tumor invasiveness is supported by our findings in asbestos-treated *Nf2* (+/-) mice, which show diminished survival due to the invasive nature of their malignant mesotheliomas, which develop at a faster rate than in WT mice.

To date, treatment of human malignant mesotheliomas has proven to be an enormous challenge. These tumors are resistant to current modalities, and new approaches are desperately needed.

Because the animal model of malignant mesothelioma described here faithfully recapitulates many of the molecular features of the human malignancy, it could prove invaluable for the future design of novel therapeutic strategies targeting relevant growth and survival signaling pathways (e.g., the AKT pathway) implicated in this disease.

Acknowledgments

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