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Losses of Both Products of the Cdkn2a/Arf Locus Contribute to Asbestos-Induced Mesothelioma Development and Cooperate to Accelerate Tumorigenesis

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Abstract

The CDKN2A/ARF locus encompasses overlapping tumor suppressor genes p16(INK4A) and p14(ARF), which are frequently co-deleted in human malignant mesothelioma (MM). The importance of p16(INK4A) loss in human cancer is well established, but the relative significance of p14(ARF) loss has been debated. The tumor predisposition of mice singly deficient for either Ink4a or Arf, due to targeting of exons 1α or 1β, respectively, supports the idea that both play significant and nonredundant roles in suppressing spontaneous tumors. To further test this notion, we exposed Ink4a(+/-) and Arf(+/−) mice to asbestos, the major cause of MM. Asbestos-treated Ink4a(+/−) and Arf(+/-) mice showed enhanced incidence and shorter latency of MM relative to wild-type littermates. MMs from Ink4a(+/−) mice exhibited biallelic inactivation of Ink4a, loss of Arf or p53 expression and frequent loss of p15(Ink4b). In contrast, MMs from Arf(+/-) mice exhibited loss of Arf expression, but did not require loss of Ink4a or Ink4b. Mice doubly deficient for Ink4a and Arf, due to deletion of Cdkn2a/Arf exon 2, showed accelerated asbestos-induced MM formation relative to mice deficient for Ink4a or Arf alone, and MMs exhibited biallelic loss of both tumor suppressor genes. The tumor suppressor function of Arf in MM was p53-independent, since MMs with loss of Arf retained functional p53. Collectively, these in vivo data indicate that both CDKN2A/ARF gene products suppress asbestos carcinogenicity. Furthermore, while inactivation of Arf appears to be crucial for MM pathogenesis, the inactivation of both p16(INK4A) and p19(ARF) cooperate to accelerate asbestos-induced tumorigenesis.

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Introduction

CDKN2A/ARF is among the most commonly mutated loci in human cancer, encoding two different tumor suppressors translated from alternatively spliced mRNAs. p16(INK4A) is composed of exons 1α, 2 and 3, and is designated here as INK4A (inhibitor of cyclin dependent kinase 4). Human p14(ARF) is encoded by exon 1β and alternate reading frames of CDKN2A/ARF exons 2 and 3, herein referred to as ARF (alternate reading frame). Knockout mice with targeted deletion of specific Cdkn2a/Arf exons have disrupted p16Ink4a, p19Arf or both genes [1,2,3,4] and develop a different spectrum of spontaneous tumors, although not malignant mesotheliomas (MMs). A differential impact of heterozygous loss of Ink4a or Arf to the induction of MM by asbestos has not been previously addressed. The studies presented here provide genetic evidence for the significance of Ink4a and Arf alterations in MM by directly comparing susceptibility to tumor induction by asbestos in Ink4a-deficient, Arf-deficient and doubly heterozygous Ink4a;Arf mice in a common genetic background. The enhanced tumor susceptibility of mice singly deficient for either p16(Ink4a) or p19(Arf) supports the view that both play significant and nonredundant roles in suppressing malignant transformation. The fact that mice deficient for both tumor suppressors have accelerated tumor development indicates that inactivation of both p16(Ink4a) and p19(Arf) cooperate to promote asbestos carcinogenicity.

Results

Using a genetic approach, we assessed the relative contribution of Ink4a and Arf deficiency to induction of asbestos-induced tumor...
there also was loss of lining. Most tumors in wild-type mice were early-stage MMs, occasional spheroids, and diffuse peritoneal seeding of the serosal occasionally observed. MMs frequently presented with ascites, fa
tivation of the predisposing tumor suppressor gene in all MMs appeared to remain functional intact based on their response to retained expression of Arf but did not express p53 protein. Cdkn2a/Arf locus. Tumor in Ink4a
Ink4a-MM cultures, whereas p15(Ink4b) was retained in 5 of 6 tumor cells from ascites or peritoneal lavage were tested with may not be obvious in the sampled tissues. Thus, where possible, because MMs may arise anywhere in the abdominal cavity and in doubly heterozygous mice. In a few cases, diagnosis was difficult in 6 of 7 MM samples tested. Expression of N12 and p53 was retained in nearly all MM cultures from Ink4a(+/-), Arf(+/-), and Ink4a;Arf(+/-) mice (Fig. 3).

Cytogenetic analyses were performed on two randomly-selected MM cultures from each mouse model. As in the human disease counterpart, MM cultures from Ink4a-, Arf-, and Ink4a;Arf-deficient mice typically showed numerous chromosome alterations. No consistent alteration was seen, although 3 of 6 tumors had extra copies of chromosome 19, including a MM from an Ink4a(+/-) mouse (#264), in which gain of chromosome 19 was the only abnormality observed. MM cultures from Arf- and Ink4a;Arf-deficient mice typically had numerous clonal structural rearrangements (Fig. 4A). Furthermore, aCGH analysis revealed a homozygous deletion encompassing the Cdkn2a/Arf locus (Fig. 4B).

Discussion

Previous studies have shown that Arf(-/-) and Ink4a(-/-) mice are more prone to spontaneous tumors than wild-type animals, but each less so than Ink4a;Arf(-/-) mice [8]. The in vivo carcinogenesis studies reported here evaluated the contribution of heterozygous mutations of Arf and Ink4a, as well as both tumor suppressor genes, to the induction of MM by asbestos, a well-established cause of this malignancy. In vivo genetic models were used to investigate the relative impact of Arf versus Ink4a deficiency in a common genetic background. By analogy, our findings suggest that p14(ARF), like p16(INK4A), is an important target of 9p21 deletions in human MM. Moreover, the data indicate that co-deletion of Arf and Ink4a can cooperate to accelerate tumorigenesis.

In previous studies of heterozygous Arf mice, spontaneous tumors exhibited loss of the residual wild-type Arf allele [2], consistent with a classical two-hit tumor suppressor gene. We found asbestos-induced MMs from heterozygous Ink4a, Arf and Ink4a;Arf mice required biallelic inactivation of the predisposing tumor suppressor genes, and MM were detected faster in the
The importance of these tumor suppressors is in accordance with an investigation using conditional knockout mice [9], in which adeno-Cre-mediated homozygous excision of \(\text{Ink4a}^+\) and \(\text{Arf}^+\) was sufficient to induce MM in the absence of asbestos exposure.

Notably, while tumor cells from \(\text{Ink4a}^+/-\)-deficient mice acquired loss of \(\text{Arf}\) or \(\text{p53}\) expression, loss of \(\text{p16(Ink4a)}\) was observed in only 3 of 6 MMs from \(\text{Arf}^+/-\) mice. Similarly, in our earlier studies of \(\text{Arf}\)-deficient mice in a different (C57Bl/6) genetic background, all 11 MMs analyzed showed loss of \(\text{Arf}\), although loss of \(\text{p16(Ink4a)}\) was identified in only two tumors. Collectively, these data imply that inactivation of \(\text{Arf}\) or \(\text{p53}\) may be crucial for MM pathogenesis, whereas inactivation of \(\text{p16(Ink4a)}\) is not.

Since MMs analyzed here retained expression of \(\text{p15Ink4b}\), loss of this gene is not critical for induction of MM by asbestos. In addition, our deletion mapping studies of human MMs revealed that deletions of \(\text{p15INK4B}\) occur less frequently than losses of \(\text{CDKN2A/ARF}\) and never occurred in the absence of a homozygous loss in the \(\text{CDKN2A/ARF}\) locus [5].

Also similar to our earlier study of \(\text{Arf}\)-deficient mice in a different (C57Bl/6) background, we rarely observed loss of \(\text{Nf2}\) in MMs arising in \(\text{Ink4a-}\), \(\text{Arf-}\) or \(\text{Ink4a;Arf-}\)-deficient mice. Inactivation of the \(\text{Nf2}\) tumor suppressor gene is postulated to facilitate cell cycle progression and tissue invasion/metastasis [10], and \(\text{Nf2}\)-deficient mice are predisposed to asbestos-induced MM and contribute to its invasiveness and spreading [5,11]. However, \(\text{Nf2}/\text{merlin}\) loss does not appear to be required for development of MM in mice having this genotype and/or background.

Lastly, we found retention of functional \(\text{p53}\) expression in MM cells that exhibit loss of \(\text{Arf}\) expression. Only one of the 20 asbestos-induced MMs showed loss of \(\text{p53}\) expression (Fig. 3B), and that single sample retained expression of \(\text{Arf}\), consistent with our previous work showing a reciprocal pattern of inactivation of \(\text{Tp53}\) in asbestos-induced MMs from \(\text{Nf2}^+/-\) mice that had retained expression of \(\text{Arf}\) [5,6]. Interestingly, in MM cells with loss of \(\text{Arf}\), the \(\text{p53}\) pathway appeared to remain functional based on response to DNA damage (Fig. 3C). These results from genetic model systems suggest that \(\text{Arf}\) loss can contribute to MM pathogenesis via \(\text{p53}\)-independent pathway(s), as previously noted in human MM cells [12], and that an intact \(\text{p53}\) pathway remains a potential target for the treatment of this highly aggressive, chemo-resistant malignancy.

In summary, this is the first report directly assessing the relative importance of \(\text{Ink4a}\) and \(\text{Arf}\) in the susceptibility to asbestos-induced MM. Collectively, these \(\text{in vivo}\) data indicate that both \(\text{CDKN2A/ARF}\) gene products suppress asbestos carcinogenicity. Furthermore, while \(\text{Arf}\) inactivation appears to be critical for MM pathogenesis and genomic instability (Fig. 4), the inactivation of both \(\text{p16(Ink4a)}\) and \(\text{p19(Arf)}\) cooperate to accelerate asbestos-induced tumorigenesis. Thus, future therapeutic approaches for MM should consider targeting pathways cooperatively regulated by both tumor suppressor genes.

**Materials and Methods**

### Animals and treatments

\(\text{Ink4a}^+\) (01XE4, FVB.129-Cdkn2atm2.1Rdp) [3] and \(\text{Ink4a;Arf}^+\) (01XB2, FVB/N.129-Cdkn2atm2.1Rdp) [4] mice were from the Mouse.
Models of Human Cancers Consortium. Mice lacking Arf [a gift of N. Sharpless] were generated with Cre-mediated excision of the neomycin selection cassette, similar to Ink4a mice [3]. All mice were in a comparable genetic background [8]. Mice were backcrossed at least two additional generations with FVB/N mice for uniformity and genotyped as described (MMHCC and [8]). Procedures were compliant with the NIH Guide for the Care and Use of Laboratory Animals.

6–8 week-old mice were injected intraperitoneally every 3 weeks with 400 mg crocidolite (UICC, SPI Supplies) (total, 3.2 mg/mouse), or with equivalent control TiO2 particles (Aldrich) [5,6]. Mice were scored as having MM based on histological evidence and/or if tumor cells exhibited a combination of three or more MM markers, including mesothelin, as assessed by reverse transcriptase-PCR (RT-PCR) and/or immunohistochemistry (Supplemental tables in File S1, Supplemental Fig. S1). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Fox Chase Cancer Center (protocol number: 00-26). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Immunohistochemistry

Slides of formalin-fixed, paraffin-embedded samples were incubated with antibodies against pan-cytokeratin and cytokeratin 8 (Sigma) and mesothelin (Santa Cruz Biotechnology), which were detected with biotinylated secondary antibodies. Sections were stained with DAB and counterstained with hematoxylin.

Primary cell cultures. Primary MM cells were isolated from ascitic fluid and/or peritoneal lavage, as described [5]. All primary cell cultures used for the molecular analyses were from passages ≤6. PCR analysis was conducted on all cultures that expressed mesothelial markers, and immunoblot analysis was performed on a random set of cultures to validate the PCR results. To test for p53, sub-confluent mouse MM cells (≥passage 6) were treated with Etoposide (100 μM) or UV irradiation (80 J/m²) 24 hours post-treatment.

PCR

RT-PCR was used to evaluate tumor cells for expression of E-cadherin, N-cadherin, cytokeratin 18 and cytokeratin 19 [5]. Control Gapdh was used to assess template integrity [6]. RT-PCR for mesothelin used primers 5′-ATCAAGACATTCCGTGGGTTGGG-3′ and 5′-CGGTTAAAGCAGTGGAGAAG-3′. Oligonucleotides for genomic and RT-PCR of Ink4a, Ink4b, Nf2 and p53 were as described [5,6]. Primers for p19(Arf) exon 1b were based on National Center of Biotechnology Information (NCBI) sequences.

Immunoblotting

Immunoblots were prepared with 15–30 μg of protein/sample, as described [5,6]. Antibodies included anti-Arf (Abcam), anti-p53 (NCL-p53-505, Novocastra), and anti-Ink4a (Santa Cruz).
Karyotypic and M-FISH Analysis

Preparation of metaphases and G-banding were performed as reported [13]. Guidelines for karyotypic designations of mouse metaphase chromosomes are found at http://www.pathology.washington.edu/research/cytopages/idiograms/mouse/. Metaphase preparations were hybridized using a 21Xmouse mFISH kit (MetaSystems). Image capturing/processing utilized a Zeiss AxioImager Z2 fluorescence microscope, with single band pass filters (Chroma Technology) appropriate for each fluorochrome and an Isis/mFISH image analysis system (MetaSystems).

Array-CGH

Genomic DNA was isolated from primary MM cell cultures at passages ≤6. Agilent 244K Genomic DNA Arrays and scanner were used for DNA copy number analysis. Data were extracted using Feature Extraction Software, and output was imported into CGH Analytics for DNA Copy Number Analysis (Agilent).

Supporting Information

Figure S1 Immunohistochemical staining of a MM tumor with anti-mesothelin (MSN) or anti-MSN plus blocking peptide to show specificity of staining. (TIF)

Figure S2 Retention of mutant Arf allele in MMs from Arf(+/−) mice. A, Abnormally large RT-PCR product amplified with Arf-specific primers for exon 1β. Samples are from MM cells of five Arf

Figure 4. Chromosomal analyses of MM cells from an Arf-deficient mouse. A, mFISH revealed one or more copies of 1;15, 1;18, 2;13, 5;6, and 6;14 rearrangements; der(6) composed of (from centromere to telomere) segments of chromosomes 6, 6, 6, and 4; deletion of chromosome 14; and trisomies of chromosomes 8, 10, 17 and 19. B, aCGH analysis showed gains of chromosomes 8, 10, 13, 17, 18, and 19; and deletions of two regions of chromosome 4, one of which (lower) represents a homozygous deletion encompassing the Cdkn2a/Arf locus embedded within a hemizygous deletion.
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(+/-) mice (lanes 1–5); lane 6 is from wild-type mouse embryonic fibroblasts. B, Sequencing of PCR products revealed an 84-bp insertion (grey italicized letters) in the mutated $\text{Arf}$ allele (Mu.) replacing an $\text{AG}$ (underlined) in the wild-type allele (Wt.). The insertion generates a predicted stop codon (marked in black), which would result in unsuccessful translation of the p19(Arf) protein.

(TIF)

File S1 MM Markers for primary cell cultures derived from asbestos-treated mice.

(DOC)

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