Spontaneous Uveal Amelanotic Melanoma in Transgenic Tyr-RAS+ Ink4a/Arf−/− Mice

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Objective: To characterize a murine model of spontaneous amelanotic melanoma arising in the uvea of transgenic mice bearing a targeted deletion of the Ink4a/Arf tumor suppressor locus (exons 2 and 3) and expressing human H-ras controlled by the human tyrosinase promoter.

Methods: Ocular lesions developed in 20 (15.7%) of 127 male albino Tyr-RAS+ Ink4a/Arf−/− transgenic FVB/N mice within 6 months, and were evaluated histologically and ultrastructurally.

Results: Uveal melanomas were locally invasive but confined to the eye, with no evidence of metastasis. Tumor cells exhibited epithelioid and spindle-shaped morphological features and closely resembled the human counterpart. Melan-A, S100 and neuron-specific enolase expression were detected immunohistochemically. Melanosomal structures were detected using electron microscopy. The retinal pigment epithelium was intact above small melanomas, and electron microscopy of the tumors failed to show the presence of basement membrane formation or desmosomes.

Conclusion: Spontaneous uveal malignant melanomas occurring in male Tyr-RAS+ Ink4a/Arf−/− transgenic mice arise within the choroid or ciliary body and share histopathological features characteristic of human uveal melanoma.

Clinical Relevance: Uveal melanoma research has benefited from xenograft models, but engineered mouse models of spontaneous uveal amelanotic melanoma will undoubtedly further our understanding of the genetic underpinning for this disease.


Human uveal melanoma, although a rare disease, is the most common primary intraocular cancer and accounts for 12% of all melanomas. More than 90% of uveal melanomas arise in the ciliary body and/or choroid and constitute posterior uveal melanomas; approximately 5% to 8% of uveal melanomas arise in the iris. Iris melanomas are generally smaller, with less potential for metastasis, and are treated differently than posterior uveal melanomas. Human uveal melanomas metastasize most often to the liver, as well as to the lung, brain, and other sites.

Ocular melanomas are rare spontaneous neoplasms for laboratory rodent species, but sporadic cases have been reported in dogs, cats, horses, and rats. Two principal strategies have been exploited to create ocular melanomas in laboratory animals for research purposes. Cultured human or animal melanoma cells can be implanted within the uveal tract of laboratory animals. Cultured tumor cells, however, may develop adaptive changes selected by growth in vitro that distinguish them from tumor cells in vivo. The alternative strategy exploits animal models with increased tendencies to develop ocular tumors. In 2 such model systems, in which the SV40 T antigen or the human H-ras oncogenes were expressed in transgenic mice under the transcriptional control of the human tyrosinase promoter, ocular melanomas were observed, although these lesions typically derived from retinal pigment epithelial (RPE) cells rather than uveal melanocytes.

In a different transgenic model in which the human tyrosinase enhancer/promoter fusion element was used to drive melanocyte-specific expression of activated human H-ras transgene (which was integrated into the Y chromosome), transgenic animals with Ink4a/Arf-deficient background (Tyr-RAS+ Ink4a/Arf−/−) experienced spontaneous melanoma development with high penetrance and short latency. In addition to nodular cutaneous melanomas arising in the dermis, occasional ocular melanomas were observed. To characterize these ocular lesions fully in Tyr-RAS+ Ink4a/Arf−/− animals, we performed detailed histopathological...
and ultrastructural analyses and provide evidence herein that the Tyr-RAS+ Ink4a/Arf−/− mouse is a relevant model of human uveal melanoma.

**METHODS**

**TRANSGENIC STRAINS**

The Tyr-RAS+ Ink4a/Arf+− FVB/N breeders were obtained from the Mouse Models of Human Cancers Consortium, Frederick, Md (http://emice.nci.nih.gov/). The Tyr-RAS+ and Ink4a/Arf genotypes of breeder animals and offspring were determined by polymerase chain reaction analysis, as described later. Tyr-RAS+ Ink4a/Arf−/− males, selected from the first set of litters, were bred with female Tyr-RAS− Ink4a/Arf−/− siblings to establish the inbred homozygous null Ink4a/Arf line. A total of 127 of these homozygous null Ink4a/Arf−/− animals were studied. Animals were provided water and rodent chow (NIH 31) ad libitum. Animals were housed and treated according to National Center for Toxicological Research, Institutional Animal Care and Use Committee, and American Association for Laboratory Animal Science guidelines. Affected animals were killed humanely with carbon dioxide and rehydrated in graded ethanol solutions for immunohistochemical staining. Endogenous peroxidase was quenched with 3% hydrogen peroxide containing 0.1% sodium azide. The sections were heated for 20 minutes in antigen retrieval solution (10 mM sodium citrate, pH 6.0) using a 700-W microwave oven on full power. Nonspecific binding was blocked using 0.5% casein in Tris-buffered saline solution (0.01M Tris buffer and 0.15M sodium chloride, pH 7.2), followed by incubation with mouse monoclonal anti–Melan-A antibodies (Vector Laboratories, Burlingame, Calif), or mouse polyclonal anti–S100 protein antibodies (DAKO, Carpinteria, Calif), or mouse monoclonal anti-neuron-specific enolase (NSE) (DAKO), antivimentin (DAKO), or anti–pan cytokeratins (Pan Cytokeratin Plus; Biocare Medical, Walnut Creek, Calif). The anti–cytokeratin antibody cocktail used is noted for reactivity against the CK-8 and CK-18 isoforms expressed by RPE. Antigen-specific antibody binding was detected using a routine streptavidin staining system with biotinylated goat anti–mouse IgG secondary antibodies (DAKO, Carpinteria, Calif), or mouse monoclonal anti–Melan-A antibodies (Vector Laboratories, West Grove, Pa), streptavidin-conjugated horseradish peroxidase enzyme label (Jackson Immunoresearch), and 3,3′-diaminobenzidine hydrochloride chromogen. Tissue sections were lightly counterstained with Mayer hematoxylin. Negative control analyses, in which phosphate-buffered saline was substituted for primary antibody, were performed on all specimens. Furthermore, a battery of positive and negative control tissues was stained when indicated. Most of our specimens had healthy indigenous control tissues on the same slide as the tumor. In each case, the retina was positive for NSE and the optic and peripheral nerves were positive for S100 protein and NSE. Additional negative control tissues included duodenal epithelium for S100 protein and NSE and liver for Melan-A. Additional positive control tissues used were lymph node for vimentin and skin for cytokeratin. Also, within the duodenal sections used as negative controls for S100 protein and NSE immunostaining, the parasympathetic ganglia of the myenteric plexus were positive for S100 protein and NSE. Positive and negative controls consistently validated the efficacy of each immunohistochemical marker examined.

**GENOTYPE ANALYSIS**

DNA was isolated from mouse tail clip samples using commercial kits (DNeasy; Qiagen, Valencia, Calif). Diagnostic polymerase chain reaction analyses were performed using the oligonucleotide primers given in the Table. Amplification products were detected using ethidium bromide–stained agarose electrophoretic gels.

**HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES**

Only mice with clinical ocular disease consistent with a potential neoplasm were selected from the breeding colony for histopathological examination. A complete necropsy was done on each mouse with a putative ocular tumor, including a careful gross examination of the liver and lung for possible metastasis. All detected gross lesions were examined microscopically. No gross lesions were observed in either the lung or the liver; therefore, a microscopic examination of these tissues was not done. Tissue specimens from 20 ocular tumors, collected from animals aged 13 to 23 weeks, were dissected and preserved in 10% neutral-buffered formaldehyde solution or modified Davidson fixative (30% formaldehyde, 15% ethanol, 5% glacial acetic acid, and 50% distilled water) 24 hours before embedding in paraffin. Paraffin sections were stained with hematoxylin-eosin and evaluated independently by veterinary (J.C.D., J.R.L., and R.R.D.) and ophthalmic (D.M.A.) pathologists. Other paraffin sections were stained with periodic acid–Schiff. Additional paraffin sections were dewaxed with xylene and rehydrated in graded ethanol solutions for immunohistochemical staining. Endogenous peroxidase was quenched with 3% hydrogen peroxide containing 0.1% sodium azide. The sections were heated for 20 minutes in antigen retrieval solution (10 mM sodium citrate, pH 6.0) using a 700-W microwave oven on full power. Nonspecific binding was blocked using 0.5% casein in Tris-buffered saline solution (0.01M Tris buffer and 0.15M sodium chloride, pH 7.2), followed by incubation with mouse monoclonal anti–Melan-A antibodies (Vector Laboratories, Burlingame, Calif), or mouse polyclonal anti–S100 protein antibodies (DAKO, Carpinteria, Calif), or mouse monoclonal anti–neuron-specific enolase (NSE) (DAKO), antivimentin (DAKO), or anti–pan cytokeratins (Pan Cytokeratin Plus; Biocare Medical, Walnut Creek, Calif). The anti–cytokeratin antibody cocktail used is noted for reactivity against the CK-8 and CK-18 isoforms expressed by RPE. Antigen-specific antibody binding was detected using a routine streptavidin staining system with biotinylated goat anti–mouse IgG secondary antibodies (DAKO, Carpinteria, Calif), or mouse monoclonal anti–Melan-A antibodies (Vector Laboratories, West Grove, Pa), streptavidin-conjugated horseradish peroxidase enzyme label (Jackson Immunoresearch), and 3,3′-diaminobenzidine hydrochloride chromogen. Tissue sections were lightly counterstained with Mayer hematoxylin. Negative control analyses, in which phosphate-buffered saline was substituted for primary antibody, were performed on all specimens. Furthermore, a battery of positive and negative control tissues was stained when indicated. Most of our specimens had healthy indigenous control tissues on the same slide as the tumor. In each case, the retina was positive for NSE and the optic and peripheral nerves were positive for S100 protein and NSE. Additional negative control tissues included duodenal epithelium for S100 protein and NSE and liver for Melan-A. Additional positive control tissues used were lymph node for vimentin and skin for cytokeratin. Also, within the duodenal sections used as negative controls for S100 protein and NSE immunostaining, the parasympathetic ganglia of the myenteric plexus were positive for S100 protein and NSE. Positive and negative controls consistently validated the efficacy of each immunohistochemical marker examined.

**TRANSMISSION ELECTRON MICROSCOPY**

Selected ocular tumors (n=5) were minced in 2.5% glutaraldehyde and 1.3% paraformaldehyde in phosphate-buffered saline immediately after dissection. Additional tumor-bearing mice (n=3) were killed humanely with carbon dioxide, and the right
Male Tyr-RAS+/Ink4a/Arf−/− mice (N=127) were monitored for 6 months. Spontaneous ocular melanomas occurred in 20 animals (15.7%) and cutaneous melanomas in 64 animals (50.4%) during this period. Cutaneous tumors or lymphomas were present in 6 cases of uveal melanoma. The affected eye contained the only neoplasm detected in the remainder of uveal melanoma cases (n=14). Cataracts developed in both eyes of all members of this transgenic strain between the ages of 4 and 7 weeks. This was before the onset of intraocular tumors and precluded ophthalmoscopic examination. Typically, the first clinical sign of developing uveal melanoma in these animals was enlargement of the affected eye; redness and inflammation developed later and occurred less frequently. Spontaneous uveal melanomas in male Tyr-RAS+/Ink4a/Arf−/− mice progressed rapidly to fill the entire eye with tumor cells within days after the first clinical symptoms appeared. Figure 1 shows the enlarged globe indicative of a spontaneous uveal melanoma in a 17-week-old male animal.

HISTOPATHOLOGICAL FEATURES

All intraocular tumors examined seemed to have arisen from the uveal tract in the posterior portion of the globe. Early tumors showed an intact RPE above the choroid (Figure 2A and B). Growth of early melanomas tended to be predominantly composed of spindled melanocytes, with fewer having epithelioid morphological features (Figure 2B). None of the early melanomas showed a multifocal distribution that would have suggested intraocular metastasis, as opposed to regional invasion evident in some of the advanced tumors. Advanced tumors demonstrated horizontal growth within the choroid, with extension into the subretinal space, displacing the retina. In eyes with large tumors, anterior displacement of the retina and lens with ulceration and rupture of the cornea was observed. Advanced cases involved infiltration of tumor cells through the sclera and into the peribulbar connective tissue. Metastasis was not detected in any organ examined grossly or microscopically.

Figure 3 demonstrates the typical appearance of a clinically detectable ocular melanoma. The globe was enlarged, and the posterior chamber was filled with tumor. A higher magnification (Figure 4A) demonstrated anterior displacement of the retina, which was often atrophic, with an intact layer of RPE.

Tumor cell morphological features included a mixture of the following: interlacing bundles of spindle cells, irregularly arranged stellate cells, or epithelioid cells often in clusters separated by fine fibrovascular stroma (Figure 4B). Tumor cells contained varying amounts of eosinophilic to amphophilic to lightly basophilic cytoplasm. Spindle-shaped melanocytes had either elongated narrow nuclei with finely stippled chromatin or plump round to oval nuclei with coarser more clumped chromatin. The epithelioid melanocytes were polyhedral, with abundant cytoplasm and round to oval nuclei with marginalized and coarsely clumped chromatin. Many of the latter had a prominent centrally located nucleolus. Some tumor cells contained 1 or more nuclei, which were often large, with irregular margins, and which contained prominent nucleoli.

EXPRESSION OF MELANOCYTIC MARKERS

The expression of characteristic melanoma/melanocyte antigens by putative uveal melanoma samples (n=9) was evaluated using immunohistochemical staining for Melan-A, S100 protein,NSE, vimentin, and cytokeratin (Figure 4C-L). Strong cytoplasmic Melan-A (Figure 4C) and S100 immunostaining in the cytoplasm and nucleus (Figure 4E) was detected in 1% to 5% and 20% to 95% of tumor cells, respectively. Neuron-specific enolase immunostaining was generally weak, and detected in 10% to 95% of tumor cells (Figure 4G). Sections from all of the 9 tumors were essentially negative for cytokeratin (Figure 4I). One tumor had a few randomly scattered tu-
mor cells immunopositive for vimentin (Figure 4K). Recognizable RPE was never immunopositive for cytokeratin in any of the tumors, but corneal and conjunctival epithelium was. Controls without primary antibody were essentially negative for each of the immunohistochemical markers (Figure 4D, F, H, J, and L).

ULTRASTRUCTURAL FEATURES

A transmission electron microscopic examination revealed occasional cells with 1 or more round to oval intracytoplasmic membrane-bound vesicles that contained a redundant system of internal membranes and were interpreted to be premelanosomes. Figure 5 demonstrates a representative structure that contained haphazardly arranged internal membranes forming a fingerprint pattern consistent with premelanosomes. These structures ranged from 100 to 400 nm, but averaged 200 nm in diameter. Two other electron-dense organelles/inclusions were suspected to be melanosomes in later stages of maturation. Ultrastructural characteristics of RPE, including desmosomes, basement membrane formation, and intracytoplasmic cigar-shaped melanosomes, were not observed.

COMMENT

Transgenic animal models are a valuable tool for studying the biological features of uveal melanoma, including the growth, invasion, and metastasis of these neoplasms, and provide obvious advantages not available to studies performed in vitro.7-13 Transgenic animal systems allow uveal melanoma to be studied in the context of integrated body systems, such as the immune and cardiovascular systems. The significance of individual cancer-related genes to the initiation and progression of melanoma can be studied by crossing transgenic strains bearing modified versions of the genes of interest. New drugs and novel therapeutic strategies, including gene therapy, can be evaluated effectively in appropriate transgenic animal models of uveal melanoma. In recognition of the importance of such models, a few transgenic mouse lines for the study of uveal melanomas have been developed. These transgenic lines have incorporated into the genome constructs composed of either a tissuespecific promoter (tyrosinase or tyrosinase-related protein 1 or 2) or a ubiquitous promoter (metallothionein) linked to different oncogenes (ret, ras, or tag) to promote growth of melanocytic tumors. Unfortunately, most
of the resultant tumors are either derived from RPE or mixed tumors of RPE and uveal melanoma. Both RPE and uveal tumors can occur in the same eye, and in most instances, both tumors have pigmentation. This complicates the use of these transgenic animals as a model to study uveal melanoma specifically.9,18

One of us (D.M.A.) previously participated in the development and characterization of 2 transgenic strains (Tyr-Tag A and Tyr-Tag B) in which the SV40 T and t antigens were under the control of the mouse tyrosinase gene.14 Pigmented intraocular tumors developed in both strains, with 100% penetrance at an average age of tumor onset at 1 week. Eyes were sequentially studied from the age of 1 to 20 weeks by light microscopy, immunohistochemistry, and electron microscopy. Metastases in Tyr-Tag A developed at 12 weeks and were observed subcutaneously and in the lungs and brain. Metastatic tumors in the brain, lungs, and retroperitoneal space were detected in Tyr-Tag B starting at 20 weeks. By hematoxylin-eosin staining and with immunohistochemical studies, well-established tumors were indistinguishable from choroidal melanomas, but contained areas of adenomatous or myxoid growth. Tumors were confirmed to be of RPE origin by documenting proliferation of the RPE in early stages.

**Figure 4.** Histological and immunohistochemical analysis of uveal melanomas in Tyr-RAS+/Ink4a/Arf−/− mice. A, A uveal melanoma underlying the retinal pigment epithelial cell layer (arrow). This is the same tumor depicted in Figure 3 (hematoxylin-eosin, original magnification ×20). B, Epithelioid tumor cell morphological features and cell packeting in a uveal melanoma. This is the same tumor depicted in Figure 3 (hematoxylin-eosin, original magnification ×40). C, Melan-A immunostaining in tumor cell cytoplasm (arrows) in a spontaneous uveal melanoma detected in a male Tyr-RAS+/Ink4a/Arf−/− mouse. D, Negative control for part C (Melan-A) at the same magnification. E, Early uveal melanoma. This is the same tumor depicted in Figure 2; S100 immunostaining is seen in the cytoplasm and nucleus of tumor cells. F, Negative control for part E (S100). G, A uveal melanoma; neuron-specific enolase (NSE) immunostaining is seen in tumor cells. H, Negative control for part G (NSE). I, A cytokeratin-negative uveal melanoma, but an immunopositive palpebral epithelium (arrow). J, Negative control for part I (cytokeratin) at the same magnification. K, A tumor with few cells immunopositive for vimentin. L, Negative control for part K (vimentin). In A, B, I, and J, the bar indicates 100 μm; and in C through H, K, and L, 50 μm.
and by the presence of basement membrane and desmosomes on electron microscopy.

In our study, the facts that healthy-appearing RPE was consistently intact above small tumors and that electron microscopy showed an absence of basement membrane formation or desmosomes support the diagnosis of uveal melanoma. Similar to the case of advanced tumors in Tyr-Tag mice, immunohistochemical methods were not able to unequivocally ascertain that RPE was not a tumor component in Tyr-RAS+ Ink4a/Arf−/− mice. (The healthy RPE in our specimens was not stained using pan cytokeratin–specific antibodies from either of 2 different sources, one of which included specificity for CK-8 and CK-18, although antibodies specific for these cytokeratins have been used by others to stain RPE.)

Spontaneous cutaneous and uveal melanoma developed in transgenic mice bearing homozygous deletions inactivating the Ink4a and Arf tumor suppressor genes and expressing the H-ras oncogene, controlled by a recombinant human tyrosinase enhancer/promoter element. The Tyr-RAS+ Ink4a/Arf−/− genotype was first generated in mice with a pigment, but mixed, genetic background (approximately 65% C57BL/6, 25% CBA, and 10% 129Sv). Cutaneous and uveal melanomas arising in this genetic background varied in the extent of pigmentation, with a tendency to become amelanotic. The animals used in this study were generated following backcrosses for multiple generations against albino FVB/N mice.

In mice and humans, the Ink4a and Arf tumor suppressor genes are alternative products of the Ink4a/Arf (CDKN2A) locus on human 9p21 and mouse 4[C3-C6]. Initiating from exon 1α and splicing onto exons 2 to 3, the locus encodes for p16Ink4a, the founding member of the Ink4 family of cyclin-dependent kinase inhibitors upstream of the retinoblastoma protein. Alternatively, initiating from an upstream exon 1β and splicing onto exons 2 to 3 but in a different reading frame, the locus encodes for a second product, Arf (p14Arf in humans and p19Arf in mice), that was determined to be a negative regulator of MDM2, which inhibits the p53 tumor suppressor. In human cancers, including melanoma, homozygous deletions of the 9p21 locus commonly eliminate p16Ink4a and Arf products, leading to inactivation of the retinoblastoma and p53 tumor suppressor pathways. Consistent with their tumor suppressor roles, simultaneous inactivation of p16Ink4a and p19Arf in the mouse (Ink4a/Arf−/−) leads to a cancer-prone condition. A germline intragenic deletion targeting p16Ink4a has been described in familial melanoma kindreds and has provided unequivocal evidence for a role of p16Ink4a in human melanoma susceptibility.

Rare germline disruptions of Arf exon 1β, which spare Ink4a expression, have been identified in human melanoma kindreds. However, Arf-selective abnormalities of this type were not detected in at least 3 studies of human uveal melanomas, although Arf disruptions that do not also affect Ink4a have been detected in at least 2 melanoma cell lines. Nevertheless, a study in the mouse in which targeted deletion of Arf cooperates with activated H-ras to enhance melanoma susceptibility demonstrates that Arf inactivation can contribute to melanoma development.

In our study, amelanotic uveal melanomas arising in Tyr-RAS+ Ink4a/Arf−/− mice exhibited various cellular histological features consistent with uveal melanoma, including some that mimic characteristics of epithelial and mesenchymal neoplasms. These tumors also expressed Melan-A, S100 protein, and NSE, detected immunohistochemically, and messenger RNA transcripts encoding Mitf protein, tyrosine-related proteins 1 and 2, and “silver” protein were also detected (data not shown).

Melanoma can be a diagnostic challenge, particularly amelanotic melanomas. Malignant melanocytes can take various morphological features, mimicking spindled mesenchymal cells of several types and epithelial cells with either stained or clear cytoplasm. Often, melanomas are mixed, composed of 2 or more of these cell types. Cell types in human uveal melanomas were first classified by Callender in 1931. He distinguished polygonal epithelioid melanocytes with abundant cytoplasm and coarsely clumped marginal chromatin and 2 types of spindled melanocytes: (1) spindle A with long narrow nuclei and delicate reticular chromatin and (2) spindle B with wider nuclei and coarser chromatin. The ocular tumors in our study had similar cellular characteristics, consistent with altered differentiation among tumor cell subpopulations.

Human melanoma is notorious for its metastatic proclivity. However, the metastatic phenotype has not been observed in the model described in this study. The total absence of tumors in other tissues in animals with single or multiple cutaneous melanomas renders improbable the hypothesis that uveal melanomas were formed via metastasis to the eye from cutaneous lesions. Mice that are doubly null for Ink4a/Arf (Ink4a/Arf knockout) succumb to lymphomas and fibrosarcoma with high penetrance. When interbred with the Tyr-RAS transgenic allele, the compound-mutant mice are more prone to development of primary melanoma at cutaneous or ocular sites; however, lymphoma or fibrosarcoma eventually emerges, leading to the animals’ demise. In other words, the nonmelanoma cancer phe-
notype precludes long-term follow-up of mice with primary melanoma lesions for emergence of metastatic disease. This may explain the lack of metastatic potential in this mouse melanoma model. Thus, intercross of the melanoma-prone Tyr-RAS transgenic allele onto a conditional Ink4a/Arf knock-out strain will bypass this complication and generate a model in which Ink4a/Arf function can be selectively inactivated only in the melanocytic compartment. Such a model will likely be a useful system for the study of metastatic spread of cutaneous and ocular melanoma.

In summary, the Tyr-RAS+ Ink4a/Arf−/− transgenic mouse model, to our knowledge, seems to be the first to produce uveal melanomas consistently in the absence of RPE tumors. Some epidemiologic studies10–14 associate environmental exposures with increased risk for uveal melanoma in humans; the effects of these risk factors on the frequency, latency, and progression of uveal melanoma could be evaluated in Tyr-RAS+ Ink4a/Arf−/− mice. Crossing other transgenic strains with melanoma-prone Tyr-RAS+ Ink4a/Arf−/− mice should provide greater understanding of the genetic basis for metastatic uveal melanoma. Perhaps most important, Tyr-RAS+ Ink4a/Arf−/− transgenic mice will provide a useful system for evaluating potential therapeutic or chemoprevention strategies for uveal melanoma.

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