Metastasizing Melanoma Formation Caused by Expression of Activated N-RasQ61K on an INK4a-Deficient Background

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Abstract

In human cutaneous malignant melanoma, a predominance of activated mutations in the N-ras gene has been documented. To obtain a mouse model most closely mimicking the human disease, a transgenic mouse line was generated by targeting expression of dominant-active human N-ras (N-RasQ61K) to the melanocyte lineage by tyrosinase regulatory sequences (Tyr::N-RasQ61K). Transgenic mice show hyperpigmented skin and develop cutaneous metastasizing melanoma. Consistent with the tumor suppressor function of the INK4a locus that encodes p16INK4A and p19ARF, >90% of Tyr::N-RasQ61K INK4a−/− transgenic mice develop melanoma at 6 months. Primary melanoma tumors are melanotic, multifocal, microinvasive the epidermis or epithelium of hair follicles, and disseminate as metastases to lymph nodes, lung, and liver. Primary melanoma can be transplanted s.c. in nude mice, and if injected i.v. into NOD/SCID mice colonize the lung. In addition, primary melanomas and metastases contain cells expressing the stem cell marker nestin suggesting a hierarchical structure of the tumors comprised of primitive nestin-expressing precursors and differentiated cells. In conclusion, a novel mouse model with melanotic and metastasizing melanoma was obtained by recapitulating genetic lesions frequently found in human melanoma. (Cancer Res 2005; 65(10): 4005-11)

Introduction

In the past decades, cutaneous melanoma incidence and mortality rates have been steadily increasing. The availability of mouse models retraceing the malignant nature of melanoma has helped to unveil the biology of melanoma and might contribute to ameliorate future diagnosis and therapy in the clinic (1). However, the utility of mouse melanoma models is hampered by the fact that they only rarely closely mimic the human disease and often show quite different pathology. Most melanoma models described to date are of dermal origin but lack the usual epidermal or junctional activity that characterizes the radial growth phase of human melanoma. An exception is the recently described HGF/SF transgenic mouse model for which intraepidermal lesions resembling the pagetoid spread of human melanoma was shown (2, 3).

To obtain a mouse melanoma model closely resembling the human disease, efforts are being made to introduce the same genetic stimuli. Melanoma tumors regressed when H-RasV12G expression was removed in an inducible melanoma model (13). This suggests that ras signaling is essential for initiation and maintenance of melanoma (13).

Activating mutations affecting N-ras are frequent events in early-stage melanomas. For example, 56% of congenital nevi harbored activating point mutations in codon 61 of the N-ras gene (14), and 33% of primary and 26% of metastatic clinical melanoma samples carried mutations in codons 12, 18, or 61 of N-ras (15). Moreover, in hereditary melanoma with germ line p16INK4A mutations, 95% of patients have N-ras mutated at codon 61 (16). Mutations affecting the serine/threonine kinase B-Raf are equally often found in melanoma, melanocytic nevi, and metastases (17). Importantly, B-Raf and N-ras mutations were mutually exclusive, strongly suggesting that both oncogenic activities are in the same linear pathway presumably deregulating the mitogen-activated protein kinase (MAPK) pathway. Taken together, these data indicate that activation of N-ras or B-Raf in combination with inactivation of the INK4a locus are key components in initiating and maintaining melanoma formation in human.

Here, we report the generation of a transgenic mouse line which expresses the oncogenic form of human N-ras (N-RasQ61K) in melanocytes on an INK4a-deficient background. These mice develop melanotic melanomas with high penetrance and acquire a metastatic phenotype thus mimicking the human condition.

Materials and Methods

Generation of transgenic mice. The Tyr::N-RasQ61K construct was generated using a mutant human N-RasQ61K (provided by Corlien Aarnoudse and Peter Schrier, Leiden, the Netherlands) and SV40 splice

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).
and polyadenylation sequences, cloned into the vector tyr(hs6.6/6.1)-lacZ (18). The gel-purified insert was injected into oocytes derived from superovulated B6D2F1 (F1[C57BL/6J × DBA/2] male and female matings. Tyr::N-RasQ61K transgenic mice (Tg[Tyr::N-RasQ61K]1Bee) were backcrossed to C57BL/6J mice for two generations and then mated to INK4a-deficient mice (6). The resulting mice were of a mixed genetic background crossed to C57BL/6J mice for two generations and then mated to Dct::lacZ (19) transgenic mice. For genotyping, genomic DNA was prepared from tail biopsies and analyzed by PCR with N-ras-specific primers or INK4a-specific primers (6). Reverse transcription-PCR (on tumor sample) and PCR using primers on both sides of the SV40 small T intron (in the SV40 polyA) was done as described (20).

**Histologic analysis and immunohistochemistry.** Mice were analyzed macroscopically for the occurrence of tumors, enlarged lymph nodes, and metastasis. A total of 16 melanomas and 11 enlarged lymph nodes were analyzed in detail. These samples were chosen to be representative and subsequent histologic analyses confirmed the macroscopic observations. Histology and immunohistochemistry were done using cryostat sections or paraffin sections. αPEP (provided by Vincen Herring, Laboratory of Cell Biology, National Institutes of Health, Bethesda, MD), anti-nestin (Developmental Studies Hybridoma bank, University of Iowa) were used as primary antibodies followed by Cy3-coupled (Jackson ImmunoResearch, West Grove, PA) and biotinylated (Amersham Biosciences Europe, Freiburg, Germany) donkey anti-rabbit IgG as secondary antibodies. Fast Red substrate (Sigma, St. Louis, MO) was used as the final chromogen and hematoxylin was used as the nuclear counterstain. lacZ-stained cryostat sections (21) were counterstained with nuclear red.

**Primary tumor cells and tumorigenicity.** Tumors were dissociated with collagenase I (1% in PBS) and cultured in primary melanocyte medium (22) or MAPC’s medium (23). Western blots of tumor cells were done according to standard procedures using mouse monoclonal anti-phospho-ERK1/2 (Sigma) and anti-ERK1/2 (New England Biolabs, Beverly, MA) antibodies. To test for tumor formation, cells were maintained in culture for 5 days, counted and injected s.c. into 5-week-old nude mice (Foxn1nu, ISREC breeding colony, in one experiment on polymethylmethacrylate plugs in the ear). Tumors from all mice were analyzed in detail. As expected, transgenic N-RasQ61K mice on an albino background, transgenics on the SV40 background. Albino Tyr::N-RasQ61K transgenic mice (Fig. 1D) show disruption of hair follicle architecture, inflammation, and epidermal hyperplasia as observed in the pigmented mice (data not shown). Because this phenotype had not been described in other mouse models with hyperpigmentation, it was necessary to show that this phenotype originated from N-ras-mediated alterations in the melanocyte lineage itself. Thus, skin melanocytes were removed from Tyr::N-RasQ61K transgenic mice by a toxicophagic approach expressing attenuated diphtheria toxin-A (Tyr::DT-A; ref. 18). Double transgenic mice were similar to Tyr::DT-A transgenic mice (Fig. 1D), and skin seemed normal (data not shown), which suggests, that the skin phenotype is not caused by promiscuous expression of N-ras in dermis or epidermis but due to alterations in the melanocyte lineage. In conclusion, expression of N-RasQ61K in the melanocyte lineage leads to hyperpigmentation, and persistence of melanocytes in dermis and epidermis.

**Melanoma susceptibility of Tyr::N-RasQ61K mice.** In human melanoma, the INK4a locus is frequently inactivated. Thus, Tyr::N-RasQ61K transgenic mice were crossed with INK4a knockout mice and >50 mice (INK4a+/−, INK4a−/−, wild type) were monitored for >1 year for development of melanoma and signs of morbidity and metastases (Fig. 2; Supplementary Table S1). Seventeen of 18 Tyr::N-RasQ61K INK4a−/− mice (94%) developed cutaneous melanomas within about 6 months of birth (6.8 ± 1.3 months). The incidence rate was slightly lower in Tyr::N-RasQ61K INK4a−/− mice (83%, 15 of 18 mice) but dropped to 29% (4 of 14) in Tyr::N-RasQ61K wild type at INK4a. In these latter two groups, the average latency for mice developing melanoma was ~1 year (INK4a−/−, 11.5 ± 1.8 months; wt, 12.1 ± 4.8 months; Fig. 2). The lower incidence and longer latency in mice heterozygous at the INK4a locus would suggest that melanoma formation is most likely due to the loss of heterozygosity of the INK4a tumor suppressor gene.

Macroscopically, melanoma tumors presented as black, frequently, ulcerated nodules. They occurred often simultaneously in multiple anatomic sites, involving trunk (81% of mice), the upper and lower extremities (22%), and head and neck (39%; n = 36 mice). Acal melanomas and melanomas of the genitalia were observed in a few cases. Histologically tumors were predominantly melanotic and epitheloid, contained numerous macrophages and were well vascularized. They were symmetrical with sharp circumscription and invaded the reticular dermis, subcutis and in some cases the underlying muscle (Fig. 2A). Tumors stained positive for tyrosinase (Fig. 2A) confirming their melanocytic origin. In some of the tumors, atypical melanocytes were identified in the surface or
adnexal epithelium. In certain cases, they formed nests and/or were present within a rather hyperplastic epidermis or at the epidermal/dermal junction of hair follicles (Fig. 2 A), indicating that Tyr::N-RasQ61K melanocytes have the capacity to microinvade the epidermis. Other tumors filled the lower two thirds of the dermis and did not affect the papillary dermis or the epidermis (data not shown). In all animals, the melanocytic expansion affected the follicular epithelium and surrounding dermis to a much greater extent than the interfollicular epithelium. In conclusion, Tyr::N-RasQ61K INK4a−/−C0 mice develop melanoma in the dermis and/or epidermis with a high incidence (>90%) and short latency (6 months).

Melanomas in lymph nodes of Tyr::N-RasQ61K transgenic mice. In a number of human melanoma patients, a population of melanocytes is detected in sentinel lymph nodes (25). Interestingly, in lymph nodes of tumor-bearing Tyr::N-RasQ61K transgenic mice this population was also seen (Fig. 3B). These melanocytes showed the same morphology as those present in the dermis and were present in the subcapsular and medullary sinuses and associated with melanin-laden macrophages. Melanomas were equally identified in lymph nodes (Fig. 3B), and these differed from the hyperplastic melanocytes in forming compact hypercellular nodules that excluded lymphocytes and contained only rare macrophages. Like the melanomas situated in the skin they often had an epithelioid morphology. Overall, 64% of tumor-bearing mice were diagnosed with enlarged lymph nodes, notably observed in the regional lymph nodes draining the site of melanoma formation.

Melanomas appearing in Tyr::N-RasQ61K transgenic mice give rise to liver and lung metastasis. Thirty-six percent of mice with melanoma showed lung or liver metastases, frequently associated with enlargement and pigmentation of lymph nodes. Pulmonary metastases presented as solitary pigmented nodules (Fig. 3C) and often two to three small nodules were detected at autopsy. Lung and liver metastases uniformly express S100, whereas tyrosinase immunoreactivity was much more heterogeneous (Fig. 3C and D). In conclusion, Tyr::N-RasQ61K transgenic mice develop melanomas, which metastasize to lung and liver.

Tumor formation and experimental metastasis by tumor cells ex vivo. Cells isolated from primary melanomas were able to form colonies in soft agar (data not shown) suggesting that they are capable of forming tumors when injected into immunocompromised mice. When 5 × 10⁵ cells (in three
In this study, we have established a novel mouse model for melanoma, and we show for the first time that overexpression of a mutant form of human N-ras (N-ras<sup>Q61K</sup>) in melanocytes results in a hyperpigmented skin phenotype and increases the metastatic behavior on an INK4a/C0 background. Similarly, melanocyte-specific expression of an oncogenic form of H-ras (H-ras<sup>Y120C</sup>) on an INK4a<sup>−/−</sup> background induced melanomas with comparable latency and penetrance; however, in contrast to the model presented here, these remain amelanotic and nonmetastatic (11). Although N-ras<sup>Q61K</sup> and H-ras<sup>Y120C</sup> transgenic constructs contain all the known tyrosinase regulatory elements including the distal control region (18), the melanoma phenotype might still be influenced by transgene expression or the integration site. However, the hyperpigmentation phenotype in the Tyr::N-ras<sup>Q61K</sup> line was intermediate compared with the other four founder mice generated but similar to the hyperpigmentation reported in the Tyr::H-ras<sup>Y120C</sup> mice, suggesting a similar ras activity in both models. On the other hand, the phenotype might be explained by differences between N-ras and H-ras. The ras proteins differ in their intracellular trafficking (29) and are compartmentalized in distinct plasma membrane domains, with N-ras for example present in lipid rafts, whereas cavedae rather contain H-ras, and both H-ras and N-ras are colocalized at the membrane (30). Activated K-ras and H-ras have been shown to modulate radiation sensitivity of cells very differently, most probably mediated by differential activation of phosphatidylinositol-3 kinase/Akt and MAPK pathways (31). Thus, either the exact location of the activating mutation on the Ras protein (H-ras<sup>Y120C</sup> versus N-ras<sup>Q61K</sup>) could be important, or, more likely, N-ras and H-ras activation in melanocytic cells has slightly distinct consequences either on MAPK activation or on a thus far unidentified pathway causing the different tumorigenic and metastatic potential. Phenotypically, changes in the epidermis, including reddening of the skin and inflammation, have been observed in the transgenic Tyr::N-ras<sup>Q61K</sup> mice of the experimental line (Fig. 1D) and in one of the other, now extinct lines, when bred to albino. This inflammation might be generated by melanocyte expression of cylooxygenases and prostaglandins (32) and might contribute to tumor progression and metastatic behavior (33, 34).

The Tyr::N-ras INK4a<sup>−/−</sup> mouse is a unique genetic model in which expression of the human oncogene in melanocytes reproduces the process of melanoma malignancy and metastatic capacity. With respect to histopathology and molecular pathogenesis, the model resembles human cutaneous malignant melanoma thereby making it an attractive melanoma and tumor model. First, the abnormal distribution and proliferation of melanocytes observed in newborn transgenic mice is reminiscent of congenital melanocytic nevi. In human, such congenital nevi include a band-like distribution of melanocytes in the upper dermis, with the presence of melanocytes around adnexal structures and between collagen bundles in the reticular dermis and with some cells above the basal cell layer of the epidermis (35, 36). Assuming that congenital nevi are regarded as a potential melanoma premalignant stage (particularly if of large size; ref. 37), and that N-ras mutations have been frequently found in congenital nevomelanocytic nevi (14), we suggest that the model presented here recapitulates this lesion which frequently progresses into malignant melanoma.

In addition, Tyr::N-ras<sup>Q61K</sup> transgenic mice develop multiple melanotic and highly invasive primary melanoma tumors. The development of multiple primary tumors is also characteristic of familial cutaneous melanoma with germ line INK4a mutations (38). In addition, some tumors depict nests of melanocytes along and above the dermal-epidermal junction. This junctional activity was most prominent in the follicular epidermis and sometimes extended to the overlying interfollicular epidermis, although given the presence of hyperplastic melanocytes in the dermis it remains possible that these are the real precursors of invasive tumors. In certain cases, the epidermis was not affected and the tumor seemed to originate from the dermis. Such a nonepidermal origin
of melanoma has been described in human for very large congenital nevomelanocytic nevi.

Furthermore, we have shown that 64% of Tyr::N-RasQ61K mice with primary melanomas also had melanocytic/melanoma deposits in lymph nodes. Given the presence of hyperplastic melanocytes in the lymph nodes, it remains unclear whether these tumors were of primary or metastatic origin. Hematogenous metastases of melanomas were clearly present with 36% of animals bearing cutaneous melanoma developing tumor deposits (often multiple) in lung and liver. In the experimental metastasis assay, tumor cells efficiently colonize the lungs of NOD/SCID mice and are capable of reaching other organs.

Finally, we have shown that the melanomas and metastases are heterogeneous. Only a fraction of the tumors expressed tyrosinase, a marker for differentiated melanin producing cell types such as melanocytes. The tumors also contained cell types expressing the neuronal stem cell marker nestin suggesting that they are comprised of melanin expressing cells as well as undifferentiated neural crest stem/progenitor cell types. This indicates that in this mouse model the melanomas are hierarchically structured, in agreement with recent findings suggesting that tumors may contain a minority of so-called “cancer stem cells,” which are essential for its initiation, expansion and maintenance (26, 28). The mouse model can now be used to elucidate whether nestin expression correlates with malignancy and whether melanoma stem cells exist. Interestingly, human melanomas have also been reported to contain nestin expressing cells (39) suggesting that it might be a general characteristic of melanomas, further confirming the similarity between experimental mouse tumors and those known from the clinic. Further support for the presence of melanoma stem cells was obtained by fluorescence-activated cell sorting Hoechst-negative cells (40). Initial results suggest that only 100 of these cells, that show...
“side population” characteristics, were sufficient to generate melanomas if grafted s.c. into athymic nude mice.3 In conclusion, by introducing the genetic lesions frequently found in human melanoma patients, we have been able to generate a mouse model developing metastasizing melanoma at high frequency closely resembling the human disease. These animals will not only be useful for preclinical testing of novel therapeutic approaches but should also allow the study of the molecular and cellular basis of tumor progression from the premalignant-stage to full-blown melanoma including metastasis formation.

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3 Unpublished data.

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