Distinct malignant behaviors of mouse myogenic tumors induced by different oncogenetic lesions

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Rhabdomyosarcomas (RMS) are heterogeneous cancers with myogenic differentiation features. The cytogenetic and mutational aberrations in RMS are diverse. This study examined differences in the malignant behavior of two genetically distinct and disease-relevant mouse myogenic tumor models. Kras; p1619null myogenic tumors, initiated by expression of oncogenic Kras in p16p19null mouse satellite cells, were metastatic to the lungs of the majority of tumor-bearing animals and repopulated tumors in seven of nine secondary recipients. In contrast, SmoM2 tumors, initiated by ubiquitous expression of a mutant SmoM2 allele, did not metastasize and repopulated tumors in 2 of 18 recipients only. In summary, genetically distinct myogenic tumors in mice exhibit marked differences in malignant behavior.

Keywords: rhabdomyosarcoma, myogenic differentiation, metastasis, transplantation

INTRODUCTION

Rhabdomyosarcomas (RMS) are heterogeneous cancers with myogenic differentiation (1). Fusion-positive RMS tumors carry exclusive chromosomal translocations at t(2;13)(q35;q14) or t(1;13)(p36;q14) and exhibit aggressive clinical behavior (2, 3). The remaining, fusion-negative spectrum of human RMS comprises a diverse group of tumors with frequent RAS pathway activation (4, 5) and variable mutations, including loss of heterozygosity at the PTCH1 locus (6, 7) in a subset of fusion-negative RMS. PTCH1 serves as a Hedgehog (Hh) receptor, and loss of PTCH1 function results in de-repression of downstream Hh pathway signaling. The contributions of RAS-relevant oncogenic pathways, including Ras and Hh signaling, to myogenic tumor formation were previously tested in mice (8, 9). This report highlights the distinct phenotypes of two mouse myogenic tumor models — those initiated by combined Cdkn2a (p16p19) disruption and Kras expression in transplanted muscle satellite cells (10) and those arising in the skeletal muscle of mice with activated Hh signaling due to expression of a mutant, constitutively active SmoM2 (SmoM2) allele (11, 12). We demonstrate significant differences in tumor-repopulating activity and prevalence of lung metastases between Kras-driven and Hh-driven myogenic tumors in mice. These observations reveal marked differences in malignant behavior between genetically distinct mouse myogenic tumors, suggesting that an understanding of the distinct oncogenetic underpinnings of tumors on the fusion-negative RAS spectrum may be informative for clinical prognosis and treatment.

MATERIALS AND METHODS

MICE

R26-SmoM2 (mixed genetic background including 129/Sv and Swiss Webster as main components) (11), CAGGS-CreER (11), and NOD.CB17-Prkdcscid/J (NOD.SCID) mice were purchased from The Jackson Laboratory. p16p19null mice (B6.129 background) were obtained from the NIH/Mouse Models of Human Cancer Consortium. Mice were bred and maintained at the Joslin Diabetes Center Animal Facility. All animal experiments were approved by the Joslin Diabetes Center Institutional Animal Care and Use Committee.

SARCOMA INDUCTION

Kras; p16p19null myogenic tumors were initiated by fluorescence-activated cell sorting of p16p19null satellite cells, followed by lentiviral transduction to introduce oncogenic Kras(G12v) and implantation in the gastrocnemius muscles of NOD.SCID mice as previously described (10). R26-SmoM2:CAGGS-CreER were injected with Tamoxifen (1 mg/40 g) on postnatal day 10 to activate expression of CRE recombinase and SMOM2. R26-SmoM2:CAGGS-CreER spontaneously developed multifocal skeletal muscle tumors (SmoM2 tumors) as previously described (11, 12).

HISTOPATHOLOGY

Tumor tissue was dissected, fixed in 4% paraformaldehyde for 2 h, and embedded in paraffin. Standard H&E stained sections were prepared. Staining for Actin (Dako, M0635, 1:200), Desmin (Dako, M0760, 1:50), and Ki67 (Vector Labs, VP-K451, 1:250) was performed as previously described (10).

LUNG METASTASES

Tumor-bearing mice were monitored at least twice weekly for health problems, and were sacrificed once tumors reached a volume of 1 cm³ or were ill. Lungs were dissected, fixed in 4% paraformaldehyde for 2 h, and embedded in paraffin.
As previously reported (12), the Ki67 index of p16p19
Kras; tases in humans. To assess the metastatic potential of
DIFFERENT METASTATIC POTENTIAL
than that observed in Kras; p16p19
null
19.1
many tumor cells (more than 75% of all tumor cells; Figure 1B
lacked cellular atypia and diffusely expressed desmin and actin in
cytoplasm interspersed with small round cells. SmoM2
contained many multinucleated, elongated cells with abundant
and actin (expressed terminal muscle differentiation markers such as desmin
ucleated giant cells. Subsets of cells (<50% of all tumor cells)
atypical nuclei, frequent mitotic figures, and occasional multinucleated giant cells. Subsets of cells (<50% of all tumor cells)
expressed terminal muscle differentiation markers such as desmin and actin (Figure 1A), and the proliferative index as evidenced by
the percentage of Ki67-expressing nuclei was 41.6 ± 12.5% (range
30.5–59.3%; four tumors evaluated) (Table 1). SmoM2 tumors
contained many multinucleated, elongated cells with abundant
cytoplasm interspersed with small round cells. SmoM2 tumors
lacked cellular atypia and diffusely expressed desmin and actin in
many tumor cells (more than 75% of all tumor cells; Figure 1B).
As previously reported (12), the Ki67 index of SmoM2 tumors was
19.1 ± 15.9% (range 3.4–41.8%; six tumors evaluated) and lower
than that observed in Kras; p16p19null tumors (p = 0.05; Table 1).

Kras; p16p19null AND SmoM2 MOUSE MYOGENIC TUMORS HAVE
DIFFERENT METASTATIC POTENTIAL
The lung is the primary organ affected by distant sarcoma metas-
tases in humans. To assess the metastatic potential of Kras;
p16p19null and SmoM2 tumors, random lung sections obtained
from tumor-bearing animals were screened for the presence of
metastases by Roderick T. Bronson.

TUMOR TRANSPLANTATION
Tumors were harvested, digested in DMEM + 0.2% collagenase
type II (Invitrogen) + 0.05% dispase (Invitrogen) for 90 min at
37°C in a shaking waterbath, triturated to disrupt the remaining
tumor pieces, and filtered through a 70 mm cell strainer. Red blood
cells were lysed from tumor cell preparations by 3 min incubation
in 0.15 M ammonium chloride, 0.01 M potassium bicarbonate
solution on ice. Defined numbers of tumor cells were resus-
pended in 10–15 ml of HBSS with 2% FBS and injected into
the gastrocnemius muscles of 1- to 3-month-old, anesthetized
NOD.SCID mice using a transdermally inserted dental needle
attached to a Hamilton syringe via polyethylene tubing. Recipient
muscles were preinjured 24 h before cell implantation by injec-
tion of 25 ml of a 0.03 mg/ml solution of cardiotoxin (from Naja
mossambica, Sigma) in order to enhance cell engraftment. Mice
were screened once weekly for the development of tumors at the
injection sites.

STATISTICS
Differences between Kras; p16p19null and SmoM2 mouse myogenic
tumors were evaluated by T-test (Ki67 indices), Fisher’s Exact
test (prevalence of lung metastases), and Kaplan–Meier analysis
(tumor-repopulating activity).

RESULTS
Kras; p16p19null AND SmoM2 MOUSE TUMORS EXHIBIT A MYOGENIC
TUMOR PHENOTYPE
Kras; p16p19null mouse myogenic tumors were induced by intra-
muscular implantation of Kras(G12v)-expressing p16p19null muscle
cell satellite cells (10). In contrast, SmoM2 mouse myogenic tumors
were initiated by ubiquitous activation of a mutant, constitutively
active smoothened (SmoM2) allele in R26-SmoM2;CAGGS-CreER
mice (11, 12). The phenotypes of Kras; p16p19null and SmoM2
myogenic tumors were previously described (10–12). In brief,
Kras; p16p19null tumors contained bundles of cells with large,
atypical nuclei, frequent mitotic figures, and occasional multinucleated giant cells. Subsets of cells (<50% of all tumor cells)
expressed terminal muscle differentiation markers such as desmin
and actin (Figure 1A), and the proliferative index as evidenced by
the percentage of Ki67-expressing nuclei was 41.6 ± 12.5% (range
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than that observed in Kras; p16p19null tumors (p = 0.05; Table 1).

Kras; p16p19null AND SmoM2 MOUSE MYOGENIC TUMORS DIFFER IN
TUMOR-REPOPULATING ACTIVITY
Most malignant tumors contain cells that have the capacity to
repopulate secondary tumors when transplanted into a suscepti-
ble secondary environment, and this assay has been used as a test of
the malignancy of distinct tumors and tumor cell subsets (13). To
evaluate the tumor-repopulating activity of Kras; p16p19null
and SmoM2 mouse myogenic tumors, viable tumor cells were trans-
planted into the cardiotoxin-pre-injured gastrocnemius muscles of
NOD.SCID mice. The Kras; p16p19null tumor cell pool contains
approximately 70% GFP+ cells and 30% GFP− cells (10). Because
tumor-repopulating activity in Kras; p16p19null tumors resides
within the Kras-expressing, GFP+ subset of tumor cells descended
from virally infected satellite cells (Figure S1 in Supplementary
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February 2015 | Volume 5 | Article 50 | 2
Table 1 | Differences in the malignant behavior of Kras; p16p19null and SmoM2 mouse tumors.

<table>
<thead>
<tr>
<th></th>
<th>Kras; p16p19null tumors</th>
<th>SmoM2 tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal muscle differentiation</td>
<td>Actin/desmin expression in &lt;50% of tumor cells</td>
<td>Actin/desmin expression in &gt;75% of tumor cells</td>
</tr>
<tr>
<td>Ki67 index (p = 0.05)</td>
<td>41.6 ± 12.5%</td>
<td>19.1 ± 15.9%</td>
</tr>
<tr>
<td>Metastases (p = 0.001)</td>
<td>7 of 9 mice with lung metastases</td>
<td>0 of 10 mice with lung metastases</td>
</tr>
<tr>
<td>Transplantation (p &lt; 0.001)</td>
<td>7 of 9 transplanted mice developed tumors (50 cells injected)</td>
<td>2 of 10 transplanted mice developed secondary tumors (100–150k cells injected)</td>
</tr>
</tbody>
</table>

Kras; p16p19null and SmoM2 mouse myogenic tumors exhibit profound differences in tumor-repopulating activity and metastatic behavior.

DISCUSSION

Our findings highlight differences in the malignant phenotype and behavior of mouse myogenic tumors driven by activation of distinct RMS-relevant oncogenic pathways. Kras; p16p19null myogenic tumors were metastatic to the lungs of the majority of tumor-bearing animals and contained high tumor-repopulating activity. In contrast, SmoM2 tumors did not metastasize and were substantially less effective in repopulating tumors in secondary recipients. These observations indicate that genetically distinct myogenic tumors in mice display marked differences in their malignant behavior.
The two model systems described in this study were induced by different experimental methods. SmoM2 tumors originated from Cre-mediated activation of a conditionally expressed transgenic, Kras; p16p19null mouse tumors, on the other hand, were initiated by viral transduction and intramuscular implantation of target satellite cells. We note that Kras; Tp53−/− mouse myogenic tumors (14, 15), induced by Cre-mediated activation of oncogenic hits instead of viral transduction, exhibit a phenotype that closely resembles the Kras; p16p19null mouse tumors described here. For example, Kras; p16p19null share their propensity to metastasize to the lungs of tumor-bearing animals with Kras; Tp53−/− mouse tumors (14). Nevertheless, it is possible that differences in the tumor induction strategy (such as off-target effects of viral transduction) could contribute to the observed differences in malignant behavior between SmoM2 and Kras; p16p19null mouse myogenic tumors.

Similar to mouse myogenic tumors, human fusion-negative RMS comprises a group of tumors with clear differences in histology, myogenic differentiation state, oncogenic pathway activation, and genetic background. In recent years, subsets of human RMS tumors that exhibit a combination of specific genetic and phenotypic characteristics were distinguished. For example, a subset of human fusion-negative RMS with spindle cell/sclerosing histology was recently found to exhibit diffuse MyoD expression, carry frequent somatic MyoD mutations, and portend a poor prognosis (16, 17). Also, children with TP53 germline mutations are predisposed to develop anaplastic RMS at a young age (18), and germline mutations in DICER1 were linked to a genetic susceptibility to develop RMS of the genitourinary tract (19). Future extended (epi-)genotype/phenotype correlations might pinpoint clinically/biologically distinct subgroups of human fusion-negative RMS and identify biomarkers to facilitate prognostication and/or stratification of therapy.

AUTHOR CONTRIBUTIONS

SH, RB, and AW conceived experiments, analyzed data, wrote, and approved of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fonc.2015.00050/abstract

REFERENCES

1. Parham D, Pathologic classification of rhabdomyosarcomas and correlations with Cre-mediated activation of a conditionally expressed transgene. Kras; p16p19null mouse tumors, on the other hand, were initiated by viral transduction and intramuscular implantation of target satellite cells. We note that Kras; Tp53−/− mouse myogenic tumors (14, 15), induced by Cre-mediated activation of oncogenic hits instead of viral transduction, exhibit a phenotype that closely resembles the Kras; p16p19null mouse tumors described here. For example, Kras; p16p19null share their propensity to metastasize to the lungs of tumor-bearing animals with Kras; Tp53−/− mouse tumors (14). Nevertheless, it is possible that differences in the tumor induction strategy (such as off-target effects of viral transduction) could contribute to the observed differences in malignant behavior between SmoM2 and Kras; p16p19null mouse myogenic tumors.

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