A Novel Mouse Model for De novo Melanoma

Mayuko Y. Kumasaka, Ichiro Yajima, Khaled Hossain, Machiko Iida, Toyonori Tsuzuki, Tamio Ohno, Masahide Takahashi, Masashi Yanagisawa, and Masashi Kato

Abstract

Nevus-associated melanomas arise from pre-existing benign lesions, but de novo melanomas can also develop in the absence of such lesions. Few studies have addressed the latter phenomenon because no animal models have been described in which melanomas clearly develop in a de novo manner. In this study, we have addressed this need in defining RFP-RET-transgenic mice (RET mice) as a mouse model for multi-step melanomagenesis that proceeds via tumor-free, benign, premalignant, and malignant stages. Melanomas from RET mice exhibited decreased expression levels of endothelin receptor B (Ednrb) compared with benign tumors. In RET mice that were heterozygous for Ednrb (Ednrb+/-;RET mice), >80% of the arising primary tumors were malignant. Life span after tumor development in the mice was significantly shorter than in RET mice. Lung metastasis after tumor development was significantly higher than in RET mice. The observed process of melanomagenesis in Ednrb+/-;RET mice, which proceeded without a pre-existing benign lesion, along with the emergent characteristics in the model after tumor development corresponded well with the formation of de novo melanoma in humans. Our findings define a novel transgenic mouse model for de novo melanoma and suggest that reduced expression of Ednrb might facilitate the development of de novo melanoma in humans.

Introduction

It has been shown histopathologically that there are two kinds of carcinogenesis in human cancers. One is multistep carcinogenesis that arises from a pre-existing benign lesion, and the other is de novo carcinogenesis that arises without a pre-existing lesion (1–4). At present, however, mechanisms for multistep carcinogenesis and de novo carcinogenesis are still largely unclear. To our knowledge, there are no melanoma animal models in which tumors have been clearly shown to be de novo melanoma.

Human melanomas develop from pre-existing benign lesions (multistep melanomagenesis) and in the absence of benign lesions (de novo melanomagenesis; refs. 5–7). However, there is very limited information about the biochemical mechanisms underlying multistep melanomagenesis and de novo melanomagenesis. This is because observation of the entire process for melanomagenesis in humans is impossible. Establishment of animal models that can be used for the study of both multistep melanomagenesis and de novo melanomagenesis may contribute to the elucidation of their pathogenetic differences.

We previously established metallothionein-I-RFP-RET-transgenic mice of line 304/B6 (RET mice) that spontaneously develop systemic skin melanosis, benign melanocytic tumors, and melanoma metastasizing to distant organs stepwise (Fig. 1A; ref. 8). In this study, we introduce RET mice with heterozygously deleted Ednrb [Ednrb(+/-);RET mice] as a novel mouse model for de novo melanoma.

Materials and Methods

Mice. RET mice (Fig. 3A), in which solitary or multiple primary dome-shaped tumors macroscopically develop on the skin (head, neck, trunk, limbs, and tail) and eyes (9, 10), were used in this study. Endothelin receptor B (Ednrb)-heterozygously deleted RET mice [Ednrb(+/-);RET mice] were newly generated in this study by crossing RET mice and Ednrb-deficient mice (11). Location and shape of tumors in Ednrb (+/-);RET mice were similar to those in RET mice (Fig. 3A). We calculated tumor volumes by the integral method based on their radius and height with calculation of hemisphere volume after choosing the largest primary tumors in the case of mice with multiple tumors. Ednrb-homozygously deleted RET mice [Ednrb (−/−);RET mice] died of Hirschsprung disease within a month. This study was approved by the Animal...
Care and Use Committee (approval no.18001) and Recombi-
nation DNA Advisory Committee (approval no. 06-01) in
Chubu University, Japan.

**Real-time PCR, immunohistochemical, and Western blot
analyses.** We biochemically and histopathologically ana-
lyzed primary cutaneous tumors and metastatic tumors
from RET mice and Ednrb(+/−);RET mice by real-time
PCR, immunohistochemical, and Western blot analyses. Re-
al-time PCR was performed by the method previously de-
scribed (12). More than 10 tumors in each tumor stage
were analyzed. The immunohistochemistry with anti–Ki67
antibody (Novocastra) and anti-Ednrb (Chemicon) antibodies
was performed using a Vectastain ABC Kit (Vector) and Vector
VIP (Vector) for colorization. Results from 90 cells from six
different samples (nuclear size and N/C ratio) and Ki67 index in benign (B, white),
premalignant (P, gray), and malignant (M, black) tumors; bars, SEM. *, P < 0.01,
significantly different from benign tumors by
Kruskal-Wallis test.

**Results**

**Histopathologic definitions of stages in the process of
melanomagenesis in RET mice.** Tumors in the benign stage
(benign melanocytic tumors) in RET mice were histopatho-
logically composed of round cells with regular round nuclei
without mitotic activity (Fig. 1B). Tumors in the malignant
stage (melanoma) were histopathologically composed of cells
with nuclei of various sizes (Fig. 1B). The tumors also had a
high level of mitotic activity (Fig. 1B) and Ki67 proliferation
index (percentage of Ki67-positive cells; Fig. 1C). Microscopic
characteristics of tumors in the newly defined premalignant
stage (premalignant tumors) were intermediate between be-
nign and malignancy (Fig. 1B–D). No lung metastases were
detected in the mice with premalignant tumors (n = 18), al-
though lung metastasis was found in 43.8% of the RET mice
with malignant tumors (n = 16). Tumor size–oriented histo-
pathologic analysis in RET mice (Fig. 4A, top) revealed that
>80% (18 of 22) of tumors with sizes of >500 mm3 were benign.
All (12 of 12) tumors with sizes of >4,000 mm3 were malignant,
whereas >80% (11 of 13) of tumors with sizes of 500 to 4,000
mm3 were premalignant (Fig. 4A, top). The entire process of
melanoma development via tumor-free, benign, premalignant, and malignant stages in RET mice (Figs. 1 and 4A) corresponded to multistep melanomagenesis in humans.

**Biochemical characterization for various stages of tumors from RET mice.** To further analyze tumor stage-oriented biochemical characters in RET mice, we selected four melanoma-related molecules [melanoma cell adhesion molecule (MCAM), E-cadherin, N-cadherin, and membrane type-1 matrix metalloproteinase (MT1-MMP)] as progression markers (Fig. 2A; refs. 13–16).

Transcript expression levels of MCAM, N-cadherin, and MT1-MMP in malignant tumors were significantly higher \((P < 0.01)\) than those in benign tumors, whereas E-cadherin expression level in malignant tumors was significantly lower \((P < 0.01)\) than that in benign tumors in RET mice (Fig. 2A). These results corresponded well with results in human melanoma (15). Transcript expression levels of MCAM, N-cadherin, and MT1-MMP in premalignant tumors were similar to those in benign tumors, whereas E-cadherin expression level in premalignant tumors was comparable with that in malignant tumors in RET mice (Fig. 2A). Taken together with the histopathologic results shown in Fig. 1B to D, these results suggest that the characteristics of premalignant tumors are intermediate between benign and malignancy.

To investigate the correlation between Ednrb and melanomagenesis in RET mice, we next examined transcript expression levels of Ednrb in tumors of various stages. Ednrb transcript expression levels in malignant tumors were significantly \((P < 0.01)\) decreased compared with those in benign and premalignant tumors (Fig. 2A). Both immunohistochemical (Fig. 2B) and Western blotting (Fig. 2C) analyses also showed reduction of Ednrb protein expressions in malignant tumors compared with those in benign tumors. These results suggest that the reduction

**Figure 2.** Stage-dependent biochemical characterization of primary tumors from RET mice. A, transcript expression levels (columns, mean; bars, SEM) of MCAM, E-cadherin, N-cadherin, MT1-MMP, and Ednrb in benign (B, white), premalignant (P, gray), and malignant (M, black) tumors from RET mice examined by real-time PCR. *, \(P < 0.01\), significantly different from benign tumors by Kruskal-Wallis test. B and C, microscopic appearance (B) and Ednrb protein expression (B and C) in benign and malignant tumors from RET mice examined by immunohistochemistry with anti-Ednrb antibody, HE staining (B), and Western blot (C). Tumors were located under the dotted lines (B; bars, 50 \(\mu m\)).
of Ednrb expression in tumors occurs with progression of tumor stage in RET mice.

**Characterization of tumors from RET mice and Ednrb-heterozygously deleted RET mice [Ednrb(+/-);RET mice].** To address the biological significance of reduced Ednrb expression levels in melanoma, we next produced Ednrb-heterozygously deleted RET mice [Ednrb(+/-);RET mice]. There were no macroscopic differences in tumors between RET mice and Ednrb(+/-);RET mice (Fig. 3A). Melanocytic tumors (Fig. 3A), which were microscopically composed of Dct- and S100-positive cells (Supplementary Fig. S1), developed in Ednrb(+/-);RET mice. Both Ednrb transcript (Fig. 3C) and protein (Fig. 3D and B) expressions in <500 mm³ tumors from Ednrb(+/-);RET mice were decreased compared with those in tumors of equivalent size from RET mice. Both Ednrb transcript (Supplementary Fig. S2A) and protein (Supplementary Fig. S2B) expressions in tumors from Ednrb(+/-);RET mice were further decreased compared with those in >4,000 mm³ malignant tumors from RET mice.

We next histopathologically compared nuclear size, N/C ratio, and Ki67 index in tumors with sizes of <500 mm³ from RET mice and Ednrb(+/-);RET mice (Fig. 3A and B). Interestingly, 68.8% (11 of 16) of <500 mm³ tumors from Ednrb(+/-);RET mice were histopathologically malignant, and the rest (5 of 16 = 31.2%) were premalignant. In contrast, 82.6% (19 of 23) of <500 mm³ tumors from RET mice were benign and 17.4% (4 of 23) were premalignant. Biochemical analysis in <500 mm³ tumors from Ednrb(+/-);RET mice and RET mice suggests malignant characteristics in tumors in Ednrb(+/-);RET mice compared with those in tumors in RET mice (Fig. 3C).

We further statistically analyzed all tumors from RET mice and Ednrb(+/-);RET mice (Fig. 4A). Different from the above-described results of tumor size–oriented histopathologic analysis in tumors from RET mice (Fig. 4A, top), most (21 of 26 = 80.8%) tumors from Ednrb(+/-);RET mice were malignant and the rest were premalignant tumors (5 of 26 = 19.2%). Although the formation of the
first tumor after birth in Ednrb(+/-);RET mice was significantly ($P < 0.0001$) later than that in RET mice (Fig. 4B), life span after macroscopic detection of the first tumor in Ednrb(+/-);RET mice was significantly ($P < 0.0001$) shorter than that in RET mice (Fig. 4C). The percentage of lung metastasis, which was confirmed by immunohistochemical analysis with anti-Dct and anti-S100 antibodies (Supplementary Fig. S3), after macroscopic detection of the first tumor in Ednrb(+/-);RET mice was significantly ($P < 0.001$) higher than that in RET mice (Fig. 4D). Thus, we showed different processes of melanomagenesis in RET mice and Ednrb(+/-);RET mice.

**Discussion**

Previous studies have shown that 20% to 25% of melanomas developed from benign melanocytic lesions with multistep progression (multistep melanoma) and that 75% to 80% of melanomas arose without pre-existing benign lesions (de novo melanoma; Supplementary Table S1; refs. 5–7). The percentage of metastases in patients with de novo melanoma was shown to be higher than that in patients with multistep melanoma, whereas age at onset of de novo melanoma was later than that of multistep melanoma in humans (Supplementary Table S1). Prognosis in the patients with de novo melanoma was worse than that in the patients with multistep melanoma (Supplementary Table S1). Because the entire process of melanomagenesis in RET mice via tumor-free, benign, premalignant, and malignant stages corresponds well with multistep melanomagenesis in humans, RET mice could be an animal model for multistep melanomagenesis. On the other hand, not only were >80% of the tumors in Ednrb(+/-);RET mice malignant but also none of the tumors in Ednrb(+/-);RET mice were benign (Fig. 4A). The characteristics of late-onset, high percentage of metastasis, and poor prognosis after tumor development in Ednrb (+/-);RET mice are compatible with those in de novo melanoma in humans. Ednrb(+/-);RET mice may be the first

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*Figure 4. Comparative study between RET mice and Ednrb(+/-);RET mice. A, correlation between stage (B, benign; P, premalignant; M, malignant) and size in tumors from RET mice (circles) and Ednrb (+/-);RET mice (triangles). Volumes of 47 tumors from RET mice and 26 tumors from Ednrb(+/-);RET mice. N.D., no detection. B, percentage of tumor-free RET mice and Ednrb(+/-);RET mice after birth. C and D, survival ratio (C) and percentage of lung metastasis-free mice (D) after detection of the first tumors in RET mice and Ednrb(+/-);RET mice. $*, P < 0.0001$ and $†$, $P < 0.001$, significantly different between RET mice and Ednrb(+/-);RET mice by log rank test (B–D).
animal model in which tumors have clearly developed in a de novo manner.

Recently, it has been reported that melanoma risk in humans was significantly increased in patients with loss of function-related mutation of EDNRB (17). In this study, we showed reduction of Ednrb expression in malignant tumors compared with that in benign tumors in RET mice (Fig. 3A, C, and D). Reduction of EDNRB activity and expression by treatment with an inhibitor of EDNRB (BQ788) greatly increased vascular endothelial growth factor expression and decreased the angiogenic suppressor gravin (18). Reduced EDNRB expression might enhance metastatic ability in tumors from Ednrb(+/-);RET mice through modulation of vascular endothelial growth factor-related and gravin-related angiogenesis, resulting in their poor prognosis after development of tumors. High metastatic ability in the mice might be correlated with poor prognosis in patients with de novo melanoma compared with that in patients with multistep melanoma. These enhancements of metastatic ability by reduced Ednrb activity and expression suggest that Ednrb has a tumor-suppressing effect. In contrast, the tumor-free stage in Ednrb(+/-);RET mice was prolonged compared with that in RET mice in this study (Fig. 4B). Lahav and colleagues also revealed that reduction of EDNRB expression by BQ788 suppressed the growth of melanoma via the promotion of apoptosis (18, 19). These suppressions of tumor growth activity by reduced Ednrb activity and expression suggest that Ednrb has a tumor-promoting effect. Thus, Ednrb might have bidirectional effects on tumor growth and metastasis.

In summary, we have developed a novel animal model for de novo melanoma in addition to the previously reported animal model for multistep melanoma. We have also shown that alteration in the expression level of one molecule (Ednrb) may have a crucial effect on the process of melanoma development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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