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Short Communication

Histopathologic features of melanocytic tumors in *Xiphophorus* melanoma receptor kinase (*xmrk*)-transgenic medaka (*Oryzias latipes*)

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Abstract: Melanocytic tumors in *Xiphophorus* melanoma receptor kinase (*xmrk*)-transgenic Carbio and HB11A strains of medaka were examined histopathologically at 7 months post-hatching. Medaka of both strains developed melanocytic tumors with a penetrance of 100%. In both strains, neoplastic cells containing intracytoplasmic melanin pigment granules showed significant invasive growth patterns. In addition, epithelioid neoplastic cells were arranged in solid nests, and spindle neoplastic cells were arranged in interlacing streams and bundles. Nuclear atypia, anisokaryosis, cellular pleomorphism, and the appearance of anaplastic giant cells containing multiple nuclei or a single nucleus were observed in neoplastic lesions in both medaka strains. However, neither strain exhibited mitotic figures or invasion of blood vessels by neoplastic cells. Based on these histopathologic findings, the tumors were diagnosed as malignant melanoma. This is the first report of detailed histomorphologic characteristics of malignant melanoma in *xmrk*-transgenic medaka.
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Key words: anaplastic giant cell, malignant melanoma, medaka, *xmrk*-transgenic

Spontaneous malignant melanomas occur in several mammalian and bird species, but their incidence and the most frequently affected sites vary between species as well as among breeds within the same species[1–8]. For instance, malignant melanoma is relatively common in dogs and horses, but it is rare in cats, pigs, and cattle[2]. Whereas the most common site of malignant melanoma in canines is the oral cavity, the majority of malignant melanomas in horses occur in the skin[2, 6]. In horses, malignant melanomas are most frequently encountered in aging grey horses, and the tumors arise most commonly in the perineal region, the ventral surface of the tail, the male genitalia, and the parotid area[2, 6].

Spontaneous melanoma also occurs specifically in certain hybrid genotypes of *Xiphophorus* fish[9], in which overexpression of a mutated version of an epidermal growth factor receptor called the *Xiphophorus* melanoma receptor kinase (*xmrk*) causes the development of malignant tumors[9]. Schartl *et al.* expressed the *xmrk* gene under the control of a pigment cell-specific promoter, microphthalmia-associated transcription factor (*mitf*), in Carbio, CabR', albino (i-3), and HB32C strains of medaka[10]. These transgenic medaka developed pigment cell tumors with a penetrance of 100%, and different types of pigment cell tumors, namely, invasive melanoma, uveal melanoma, or xanthoerythrophoroma, occurred depending on the genetic background[10]. However, detailed histomorphologic characteristics of pigment cell tumors in medaka harboring the *xmrk* transgene, such as the shape, arrangement, mitotic rate, invasion into blood vessels of neoplastic cells, and degree of secondary changes such as ulcers and necrosis, have not been clarified.

In the present study, melanocytic tumors occurred in the Carbio strain and HB11A strain of *xmrk*-transgenic medaka at 7 months post-hatching. These tumors were examined histopathologically, and their histomorphologic characteristics were compared with those of melanocytic tumors in other animal species. The purpose of the present study was to compare and contrast histopathologic findings of tumors in *xmrk*-transgenic medaka with those in other animal species and evaluate the usefulness of *xmrk*-transgenic medaka as non-mammalian alternative models for studying melanocytic tumors.

The methods for establishing the Carbio *xmrk*-transgenic medaka were performed as described previously[10]. The first transgenic melanoma medaka line was generated by injecting a construct consisting of the *Xiphophorus* melanoma oncogene cDNA, *xmrk* under control of the medaka

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mitfa promoter, into one-cell stage embryos of the Carbio strain. Injected embryos were raised to adulthood and tested for presence of the transgene construct by PCR and melanoma formation. Stable lines were produced by crossing the transgene-positive G0 fish with the wild-type Carbio strain. A transgenic (*mitf*:*xmrk*) homozygous line was obtained by intercrossing. HB11A *xmrk*-transgenic medaka were established by successive backcrossing with the HB11A inbred strain. Briefly, melanoma-bearing heterozygous males or females were selected and backcrossed with HB11A males or females until the 10th generation. Fish were maintained at 25 to 26°C with a 14:10 h (light:dark) cycle in a recirculating aquaculture system equipped with carbon filtration and biofiltration. They were fed a standard powder diet (Hikarilabo 130, Meitousuien, Aichi, Japan) and artemia nauplii (Brine shrimp eggs, Marinetech, Aichi, Japan). All experiments were performed in accordance with the guidelines of the National Institute for Basic Biology.

At 7 months post-hatching, 68 fish (Carbio strain, n=29; HB11A strain, n=39) were examined histopathologically. The fish were then euthanized using 0.003% eugenol (FA100, DS Pharma Animal Health Co., Ltd., Osaka, Japan) and fixed *in toto* in Bouin's fluid overnight before postfixation with 10% neutral buffered formalin. The fish were embedded in paraffin, cut into sagittal sections, and routinely subjected to hematoxylin-eosin staining, potassium permanganate/oxalic acid melanin-bleaching, and Fontana-Masson staining.

At 7 months post-hatching, abnormal swimming was observed in medaka of both the Carbio and HB11A strains (Carbio strain medaka, 27.6% [8 of 29 fish]; HB11A strain medaka, 15.4% [6 of 39 fish]); however, no fish exhibited a moribund condition, and there was no increase in mortality.

Carbio and HB11A strain medaka developed melanocytic tumors with a penetrance of 100% at 7 months post-hatching. In Carbio medaka harboring the *xmrk* transgene, neoplastic lesions were distributed in many organs, including the skin, skeletal muscle, bone, meninges, kidneys, gills, peritoneum, intestines, ureter, urinary bladder, pleura, testis, ovary, medulla oblongata, and spinal cord (Fig. 1). The frequency of tumor distribution was particularly high in the skin (100%), skeletal muscle (93.1%), bone (89.7%), and meninges (89.7%). By contrast, in HB11A strain medaka, neoplastic lesions were more restricted in localization and only found in the skin (100%), skeletal muscle (84.6%), bone (66.7%), meninges (42.9%), and caudal fin (53.9%) (Fig. 1). Abnormal swimming was observed in the *xmrk*-transgenic medaka developing severe neoplastic lesions in skeletal muscle, bone, meninges, and caudal fin.

In both medaka strains, neoplastic cells exhibited a pattern of significant invasive growth into adjacent tissues (Fig. 2–5). In almost all medaka from both strains, proliferation of neoplastic cells was observed in the skin and body trunk musculature (Fig. 3 and 4). There were no remarkable changes in neoplastic lesions of the skin in almost all fish, and a slight degree of ulceration was observed in cutaneous neoplastic tissue in only one fish from each strain. In medaka of both strains, proliferation of neoplastic cells adjacent to bone was common (Fig. 4). In the intestines of Carbio medaka, invasive proliferation of neoplastic cells was observed in the lamina propria, submucosal tissue, and muscular layer of the mucosa (Fig. 5). In these fish, neoplastic cells infiltrated the interstitial tissue in the kidneys and proliferated in the medulla oblongata and spinal cord (Fig. 2). In neoplastic tissue of medaka of both strains, epithelioid neoplastic cells were arranged in solid packets and nests, whereas spindle neoplastic cells were arranged in interlacing streams and bundles (Fig. 6). Nuclear atypia, anisokaryosis, cellular pleomorphism, and anaplastic giant cells containing multiple nuclei or a single nucleus were observed in neoplastic lesions of both strains (Fig. 3, 7, and 8). However, no mitotic figures, no invasion of blood vessels by neoplastic cells, or necrotic changes were observed in neoplastic lesions of medaka of either strain. In Carbio medaka, the quantity of black intracytoplasmic pigment granules varied; some neoplastic cells contained numerous black intracytoplasmic pigment granules (Fig. 2), whereas others contained scarce black intracytoplasmic pigment granules (Fig. 3, 5–7). By contrast, in HB11A strain medaka, all neoplastic cells contained numerous black intracytoplasmic pigment granules (Fig. 4A).

Black intracytoplasmic pigment granules were positive for Fontana-Masson stain (Fig. 3A2 and B2), and they were bleached by the potassium permanganate/oxalic acid melanin-bleaching technique (Fig. 4B and C). Therefore, the black intracytoplasmic pigment granules were consid-

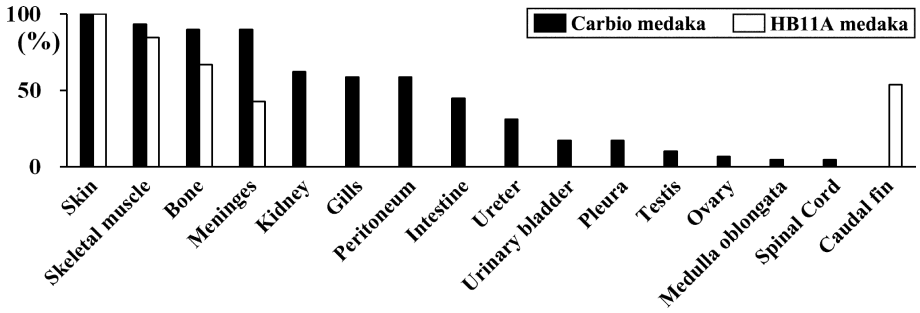

Fig. 1. Distribution of neoplastic lesions in medaka of strains Carbio and HB11A harboring the *xmrk* transgene.

ered to be melanin. These melanocytic tumors occurring in *xmrk*-transgenic medaka of both the Carbio and HB11A strains exhibited a pattern of significant invasive growth of neoplastic cells, the appearance of atypical nuclei and anaplastic giant cells, anisokaryosis, and cellular pleomorphism. Based on these histopathologic findings, the tumors were diagnosed as malignant melanoma. Mitotic figures in neoplastic cells are frequently observed in spontaneous malignant melanomas in several species of mammals and birds[1, 3, 5, 7, 8, 11–18]. According to the World Health Organiza-

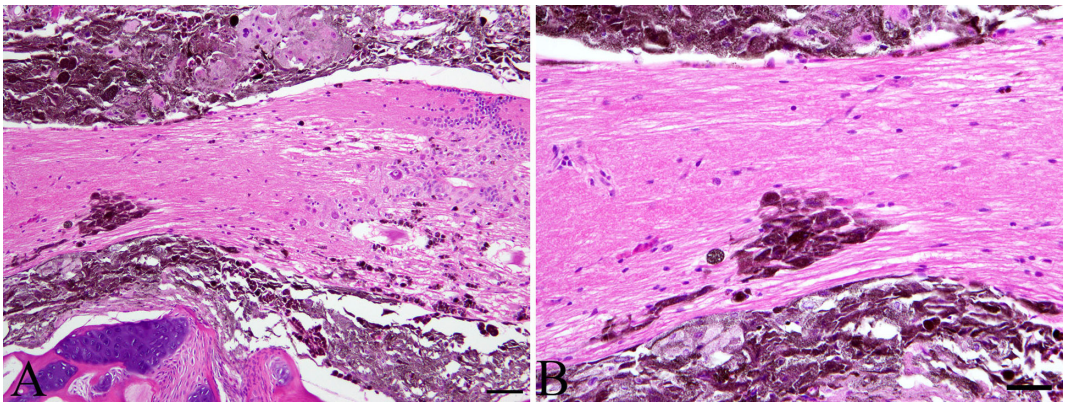

Fig. 2. Neoplastic cells containing high amounts of black intracytoplasmic pigment granules proliferated in the meninges (A, B) and medulla oblongata (B) of *xmrk*-transgenic Carbio strain medaka. Panel B shows magnified views of images shown in panel A. Hematoxylin-eosin stain without bleaching. The bar represents 50 µm (A) or 30 µm (B).

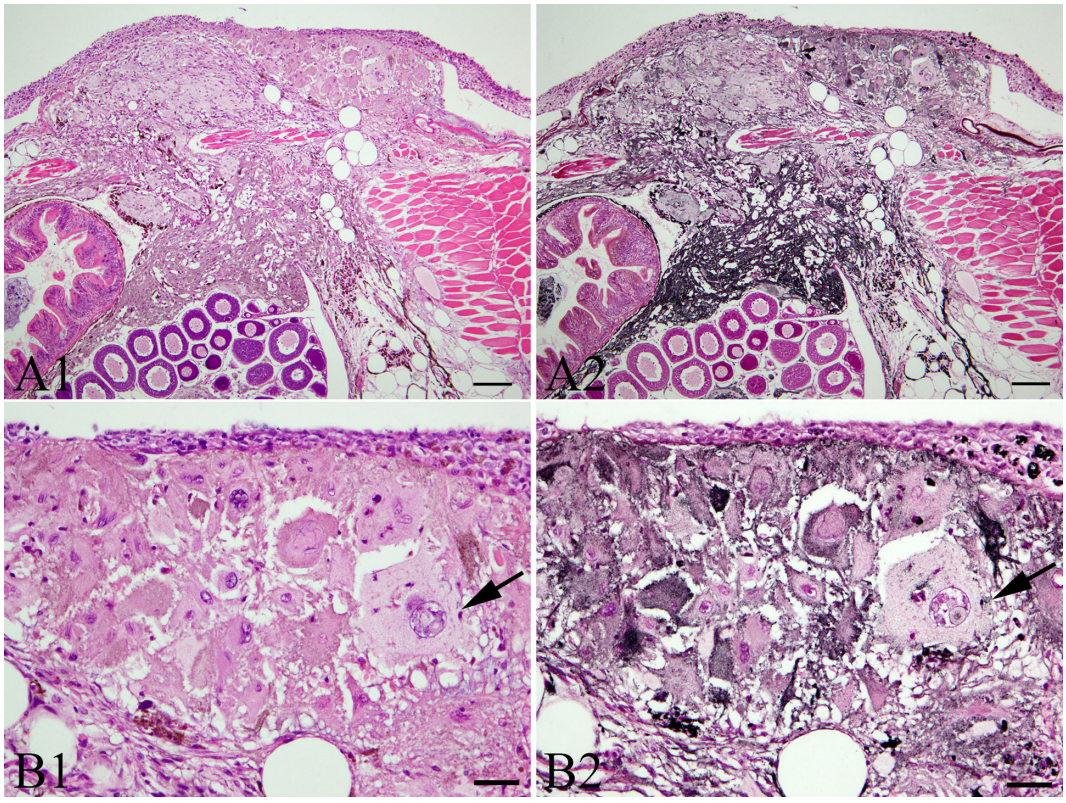

Fig. 3. Neoplastic lesions extended from the skin to the body trunk musculature and intra-abdominal organs in *xmrk*-transgenic Carbio strain medaka (A1 and A2). Neoplastic cells in the skin exhibited a variety of sizes and shapes (B1). Scarce black intracytoplasmic pigment granules were observed (B1), and they were positive for Fontana-Masson stain (B2). The arrow shows an anaplastic giant cell (B1 and B2). Panels B1 and B2 show magnified views of panels A1 and A2. 1: Hematoxylin-eosin stain without bleaching. 2: Fontana-Masson and hematoxylin-eosin double staining. The bar represents 100 µm (A1 and A2) or 30 µm (B1 and B2).

tion Histologic Classification of Epithelial and Melanocytic Skin Tumors of Domestic Animals[19], ≥3 mitotic figures per 10 high-power fields is indicative of malignancy. However, no mitotic figures were observed in the tumors of *xmrk*-transgenic medaka of either the Carbio or HB11A strain in the present study. In this respect, there is a distinct difference between the tumors of the present study and malignant melanoma in mammals and birds[1, 3, 5, 7, 8, 11–18]. The tumors in the present study exhibited a pattern of significant infiltrative growth; nevertheless, no mitotic figures were observed. A previous study demonstrated that the degree of neoplastic infiltration is not associated with the frequency of Ki-67

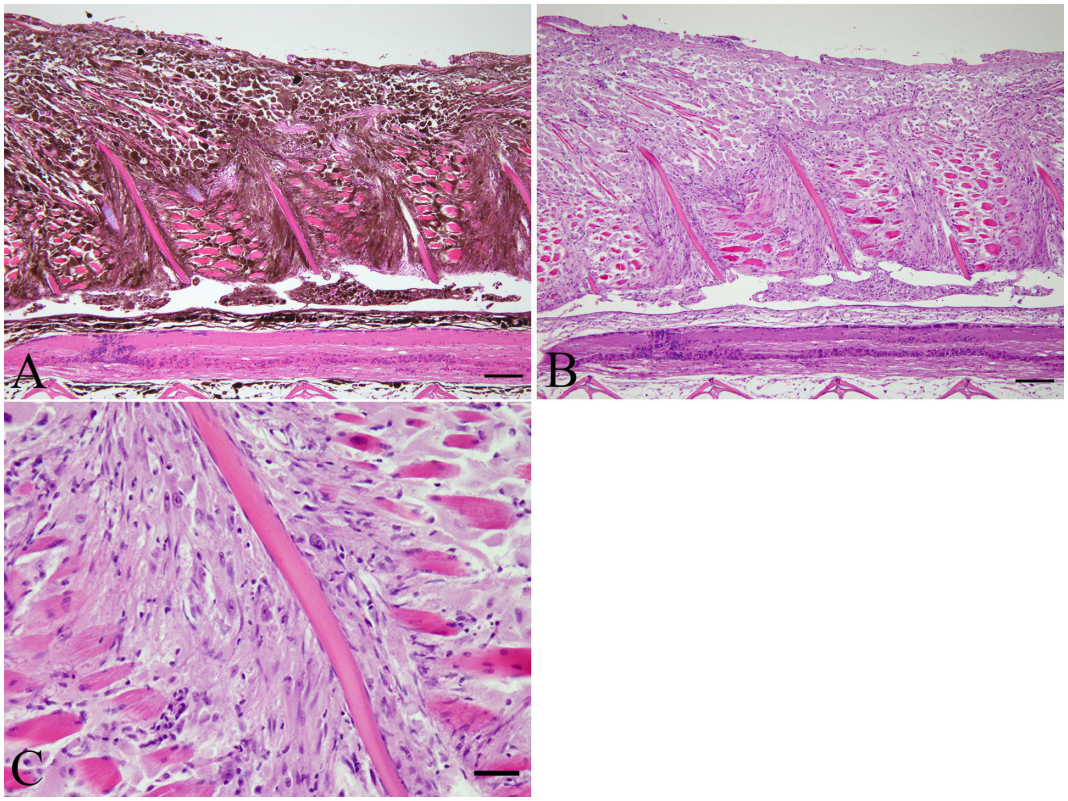

Fig. 4. Spread of neoplastic lesions from the skin to skeletal muscle and bone in *xmrk*-transgenic HB11A strain medaka (A and B). Neoplastic spindle cells proliferated adjacent to bone (C). Panel C shows magnified views of panel B. A: Hematoxylin-eosin stain without bleaching. B and C: Hematoxylin-eosin stain with bleaching. The bar represents 100 µm (A and B) or 30 µm (C).

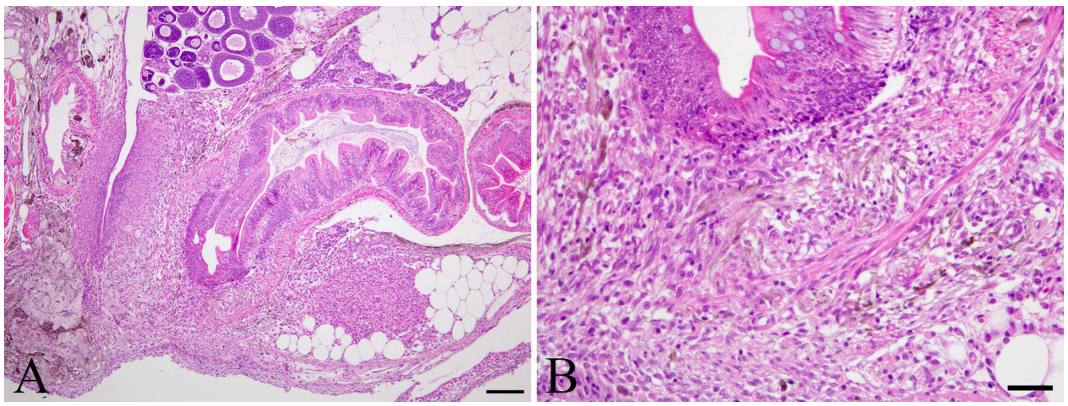

Fig. 5. Neoplastic lesions expanded from the body trunk musculature to the intestines in *xmrk*-transgenic Carbio strain medaka (A). Neoplastic spindle cells infiltrated and proliferated in the lamina propria, the submucosal tissue, and the muscular layer of the intestinal mucosa (B). Panel B shows magnified views of panel A. A and B: Hematoxylin-eosin stain without bleaching. The bar represents 100 µm (A) or 30 µm (B).

antigen expression in human malignant melanoma[20]. In human malignant melanoma, a high mitotic index and vessel invasion by neoplastic cells are recognized as prognostic factors[21–23]. These two histopathologic findings were not observed in the tumors in the present study, and this could be related to the low mortality rate under the present experimental conditions. In tumors of the present study, epithelioid neoplastic cells were arranged in solid packets and nests, and spindle cells were arranged in interlacing streams and bundles, similar to melanocytic tumors in mammals[2, 4, 6]. A previous study demonstrated that the phenotype of pigment cell tumors varied with genetic background in *xmrk*-transgenic medaka[10]. In the present study, the quantity of intracytoplasmic melanin pigment granules and the distribution of neoplastic lesions differed between the Carbio and HB11A strains, and the phenotypes of the tumors varied with the differing genetic backgrounds, similar to the results of the previous study involving *xmrk*-transgenic medaka[10]. Strain difference in expression of superoxide dismutase-3 (SOD3) gene, which acts as a tumor suppressor, occurred in rats[24, 25]. In the present study, the strain difference in the distribution of neoplastic lesions in *xmrk*-transgenic medaka may also be associated with the difference in the expression patterns of the tumor suppressor gene.

In the present study, multinucleated giant cells appeared in neoplastic lesions of *xmrk*-transgenic medaka of both the Carbio and HB11A strains. A previous study demonstrated that melanoma multinucleated giant cells may play an important role in melanoma metastasis, because they increased pulmonary metastasis ability and the β-tubulin gene group was upregulated in them[26]. Other previous studies showed that distant metastases occurred in the

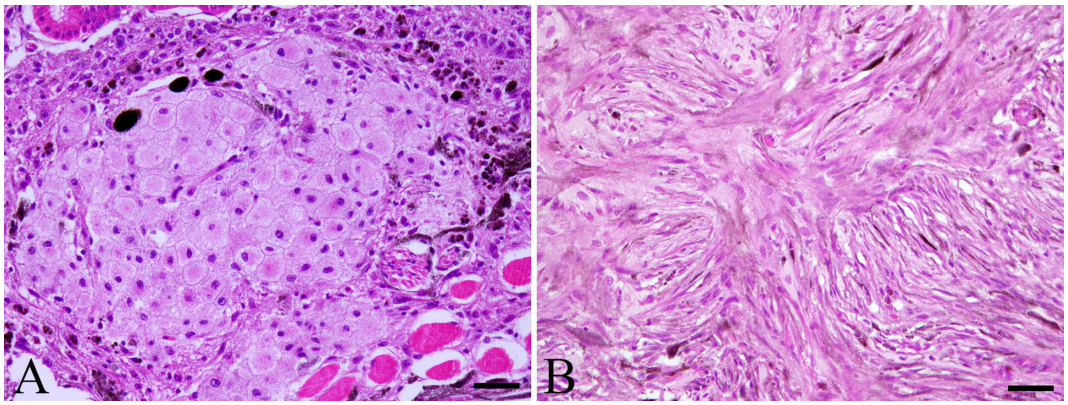

Fig. 6. Epithelioid neoplastic cells were arranged in solid packets and nests (A), and spindle neoplastic cells were arranged in interlacing streams and bundles (B) in neoplastic tissue of Carbio strain medaka harboring the *xmrk* transgene. A and B: Hematoxylin-eosin stain without bleaching. The bar represents 30 μm.

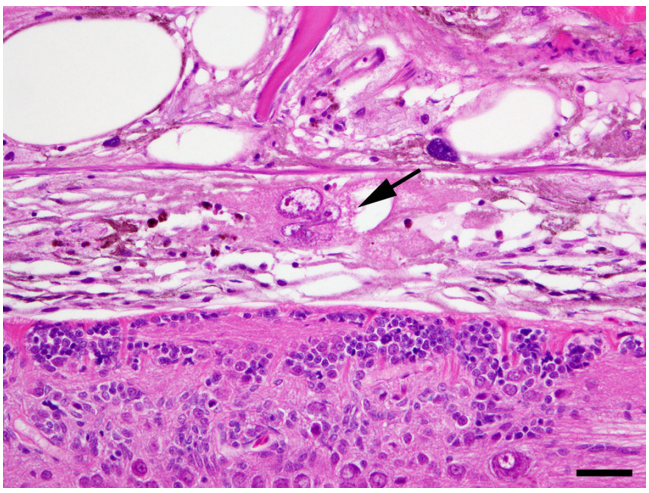

Fig. 7. Atypical nuclei appeared in neoplastic tissue of the meninges of Carbio strain medaka harboring the *xmrk* transgene (Arrow). Hematoxylin-eosin stain without bleaching. The bar represents 30 μm.

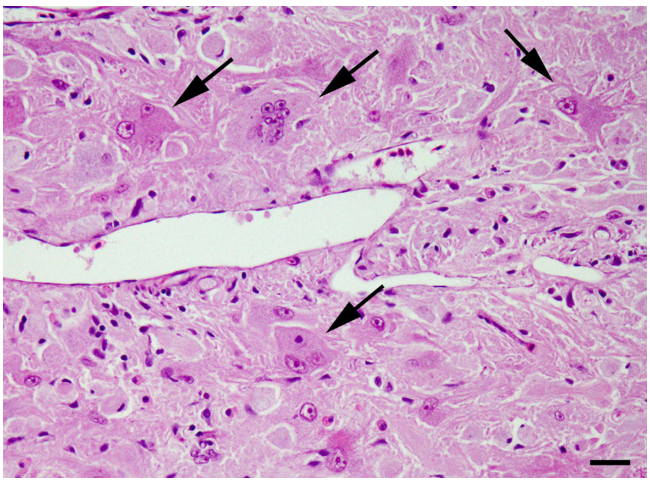

Fig. 8. Anaplastic giant cells containing multiple nuclei or a single nucleus were scattered in neoplastic tissue of HB11A strain medaka harboring the *xmrk* transgene (Arrows). Hematoxylin-eosin stain with bleaching. The bar represents 20 μm.

malignant melanomas with multinucleated giant cells of human and animals[1, 3, 27–29]. The significance of appearance of multinucleated giant cells in malignant melanoma of *xmrk*-transgenic medaka is unclear at present.

In conclusion, *xmrk*-transgenic medaka of strains Carbio and HB11A developed malignant melanoma tumors with a penetrance of 100% at 7 months post-hatching. The tumors exhibited a pattern of significant invasive growth involving neoplastic cells with no mitotic figures or blood vessel invasion. Additionally, the tumors were characterized by the appearance of anaplastic giant cells. This is the first report detailing the histomorphologic characteristics of malignant melanoma in *xmrk*-transgenic medaka.

Disclosure of Potential Conflicts of Interest: We have no conflicts of interest to declare.

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