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Differences in tumor initiation and progression of melanoma in the *Braf^{CA};Tyr-CreERT2;Pten^{f/f}* model between male and female mice.

Yougang Zhai[#], Adil J. Haresi, Lee Huang, Deborah Lang^{*}

Department of Dermatology, Boston University, Boston, Massachusetts, U.S.A.

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Dear Editor,

Cutaneous melanoma is an aggressive type of skin cancer with significant morbidity and mortality (Reed et al., 2012). To study the biology of this cancer, as well as test therapeutics, several transgenic mouse models were developed that mimic the human disease in terms of genetics, histology, and kinetics. A frequently utilized model is the *Braf^{CA};Tyr-CreERT2;Pten^{f/f}* transgenic mouse, a spatiotemporal system using the Cre-ERT2/LoxP transgene to conditionally express a mutant v-Raf murine sarcoma viral oncogene homolog B (*Braf^{CA}*) and delete exons within the phosphatase and tensin homolog (PTEN) gene simultaneously in melanocytes (Dankort et al., 2009). Melanocyte specificity is through driving expression of the CreERT2 gene with segments of the tyrosinase promoter (*Tyr-CreERT2*). For mouse models, our laboratory and others have adopted the more stringent policy of the National Institutes of Health in the consideration of sex as a biological variable (NOT-OD-15–102). This is an important initiative, since sex of the animals (as well as patients being analyzed and treated) was an often-overlooked biological variable and may cause a bias to how data are interpreted and applied.

To address this question specifically in terms of the *Braf^{CA};Tyr-CreERT2;Pten^{f/f}* mouse model, we examined tumor initiation and progression in both male and female mice. *Braf^{CA};Tyr-CreERT2;Pten^{f/f}* mice were purchased from Jackson laboratory ((Dankort et al., 2009), Jackson Laboratory stock #013590, C57BL/6J background). Mouse colonies were maintained under specific pathogen-free conditions and experimental procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC). For both sexes, mice were housed in cages of 2–5 mice from the same litter. Timepoints are characterized as induction, initiation, and progression (Figure 1A). For induction, 6–7 week-old mice were shaved on the back and 4mM 4-hydroxy-

^{*} To whom correspondence should be addressed: Deborah Lang, PhD, Boston University, Department of Dermatology, 609 Albany Street, room J205, Boston, Massachusetts, U.S.A. 02118 Telephone: 01-617-358-9721; Fax: 01-617-638-5515; deblang@bu.edu.

[#] current address: Cancer Prevention and Control Program, Fox Chase Cancer Center, Philadelphia, PA 19111

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tamoxifen (4-OHT) (Sigma Aldrich) was applied topically during three consecutive days (day 1, 2 and 3) to activate the Tyr-CreERT2 transgene and activate/inactivate the Braf and Pten alleles. The first day of induction was recorded as experimental day 1. Mice were observed daily, and melanoma initiation and progression days were recorded. The determination of tumor initiation day was when pigment cell aggregates are clearly visible from the skin surface (Figure 1A,B). The determination of progression day was that when tumor lesions form a palpable lesion by touch and form visible nodular lesions (Figure 1A,E).

Previous reports found initiation of pigmented lesions at approximately two weeks that progressed to nodular melanomas in the following two to four weeks (Dankort et al., 2009). Our findings in a pure C57B/6J background was similar, but with significant differences between sexes. The range for the first sighting of a pigmented lesion in female mice was between 13 to 21 days, and males 17 to 28 days. There were significant differences between Kaplan-Meier curves for the initiation between the sexes ($X^2= 10.97$, $p= 0.0009$ Log-rank test, Figure 1C). When graphed as a scatter dot plot, there was a significant difference in mean and median of initiation between sexes, with mean for female mice of 17.9 ± 2.3 days, and males with a mean of 22.7 ± 3.6 days ($p=0.018$, 2-tailed Mann-Whitney tests). The median time for initiation was 18 days for female mice and 22 days for male mice (Figure 1D, median indicated by line). The sex differences were more evident for progression. The lesions on the female mice progressed at a quicker pace to a palpable nodular lesion (example shown, Figure 1E) when compared to the males. The observed progression rate in female mice was between 27 to 32 days, and males 44 to 52 days. For progression, there were significant differences between Kaplan-Meier curves between the sexes ($X^2= 19.94$, $p<0.0001$ Log-rank test, Figure 1F). When graphed as a scatter dot plot, there was a significant difference in mean and median between sexes for progression, with mean for female mice of 28.3 ± 1.3 days, and males with a mean of 46.6 ± 3.5 days ($p<0.0001$, 2-tailed Mann-Whitney tests). The median time for initiation was 28 days for female mice and 47.5 days for male mice (Figure 1G, median indicated by line). The tumor development was 4-OHT dependent (Figure 1C,F grey line). After day 50, the transgenic line was prone to nonspecific tumors even in the control mice, similar to observations previously noted (Hooijkaas, Gadiot, van der Valk, Mooi, & Blank, 2012). While some of this difference in progression is due to the later initiation in the males, the difference in progression is more divergent even if this is taken into consideration (with normalization to day of initiation).

Our study supports the importance of the NIH initiative for increased stringency for considering sex as a biological variable. The majority of studies will focus on mice of only one sex, or put both sexes in one group. However, prior findings have reported sex biases, including a mouse model of Alzheimer's Disease, where the female mice demonstrated a more pronounced phenotype compared to the males (Bundy, Vied, Badger, & Nowakowski, 2019) as well as a higher susceptibility of male C57B6 mice to mycobacterium tuberculosis with corresponding greater risk of death from infection (Dibbern, Eggers, & Schneider, 2017). There is also reported sex biases in patients with melanoma. In people under 45 years old, there is a higher incidence in women compared to men, with the widest difference at 20–24 years of age (Liu et al., 2013). However, at older age groups the incidence switched, with males with the higher incidence, as well as an increased risk of metastasis and a higher

rate of mortality (Clark, From, Bernardino, & Mihm, 1969; Joosse et al., 2011; Lasithiotakis et al., 2008). It is not clear if this sex bias for melanoma is also playing a role in our model, due to our use of young mice. A link to sex hormones is supported by studies in normal melanocytes, which do not have canonical estrogen or progesterone receptors but respond to these steroids through alternative receptors such as G protein-coupled estrogen receptor (GPER) (Natale et al., 2016). Activation of GPER also inhibits melanoma progression, which may be linked to lower rates of metastasis and mortality in women (Natale et al., 2018). However, another variable may be technical, since tamoxifen is an estrogen receptor modulator and its use as an inducer in the model may have secondary effects that have differential effects in male and female mice.

In summary, in the *Braf^{CA};Tyr-CreERT2;Pten^{ff}* mouse model, female mice develop faster melanoma initiation and progression after tumor induction. These differences between female and male mice indicate that gender specific signaling/mechanisms may have impact on the development of melanoma in this model. It is an important consideration when interpreting the results based on this model system. However, this sex-dependent difference may be an added opportunity to further study sex differences in melanoma in a highly relevant and impactful mouse model.

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Figure 1. Gender difference in melanoma initiation and progression in a mouse inducible model of melanoma.

A. Mouse model kinetics. The back skin of *Brat^{CA};Tyr-CreERT2;Pten^{f/f}* mice were shaved and 4OHT (4-hydroxy tamoxifen) was topically applied for 3 consecutive days. Mice were observed daily for initiation (first sign of a pigmented lesion) and progression (palpable modular tumors) by at least two independent observers. **B,E.** Photographs of mice with initiation lesions (B) and progressed tumors (E), with magnification (2X) in insets (upper right-hand corner). **C,F.** Kaplan-Meier survival graphs of initiation (C) and progression (F) of female and male mice (red and blue lines, respectively). There were significant differences between Kaplan-Meier curves (C: $X^2 = 10.97$, $p = 0.0009$; F: $X^2 = 19.94$, $p < 0.0001$, log rank tests). The tumors were 4-OHT dependent, since melanocytic lesions did not develop in the no tamoxifen controls (grey lines). **D,G.** Scatter dot plots (median with interquartile range) of days of melanoma initiation (D) and progression (G) of female and male mice. There were significant differences between female and male mice (D: female, mean = 17.9 ± 2.3 days, male, mean = 22.7 ± 3.6 days, $p = 0.018$; G, female, mean = 28.3 ± 1.3 days, male, mean = 46.6 ± 3.5 days, $p < 0.0001$, Mann-Whitney tests, 2-tailed). For all experiments, $n = 15$ (females), $n = 8$ (males), and $n = 6$ each for male and female no tamoxifen

controls. In accordance to IACUC protocols, all mice were removed from study when tumors 1cm in diameter.

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