

Original Article

A sex-dimorphic mouse model of esophageal squamous cell carcinoma

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Abstract: Sexual dimorphism in the incidence of human esophageal cancer, including both esophageal adenocarcinoma and squamous cell carcinoma, shows male dominancy. However, the mechanisms that underlie sexual dimorphism of esophageal cancer have been understudied in vivo due to the lack of sex-dimorphic mouse models. Here, we developed a sex-dimorphic mouse model of esophageal squamous cell carcinoma (ESCC) using a lower amount of 4-nitroquinoline-1-oxide (4-NQO) and a shorter latency of tumorigenesis compared to the traditional carcinogenesis procedures. In this model, we found that male mice were highly sensitive to the tumorigenesis of ESCC whereas female mice were resistant to it. This model provided us an opportunity for investigating the mechanisms underlying sexual dimorphism of ESCC in vivo and for better understanding the sex-dimorphic incidence of ESCC in humans.

Keywords: Sexual dimorphism, mouse model, esophageal squamous cell carcinoma

Introduction

Sex dimorphism is an important feature of human cancers but has been under-investigated and mostly neglected in clinical diagnosis and therapy. More importantly, the regulatory mechanisms underlying sexual dimorphism of each cancer have been barely addressed in vivo. Cancer of the esophagus or esophageal cancer arises from the food pipe between throat and stomach. Cigarette smoking, alcohol consumption, and poor oral health are risk factors for esophageal cancer [1, 2]. Esophageal cancer is highly male-dominant in the United States and worldwide as the average incidence ratio of esophageal cancer from men to women was about 3.66 in 1975-2014 from the SEER data and 2.90 in 2012 from the World Cancer Report data. Sex hormone signaling was found to be involved in the regulation of sex disparities in esophageal cancer with mixed results and all of these studies were completed in human cancer cell lines [3-13]. There is barely any study on sexual dimorphism of esophageal cancer in vivo. A key reason is due to the lack of in vivo mouse models with sexual dimorphism of esophageal cancer. Esophageal cancer has

two major subtypes: esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC) and both subtypes show male dominancy. In this study, we will mainly focus on ESCC.

Here, we developed a sex-dimorphic mouse model of ESCC using a lower amount of 4-nitroquinoline-1-oxide (4-NQO) and a shorter latency of tumorigenesis compared to traditional 4-NQO approaches. In this model, we found that male mice were highly sensitive to the tumorigenesis of ESCC whereas female mice were resistant to it. This model provided us an opportunity to investigate the mechanism underlying sexual dimorphism of ESCC in vivo in the future.

Methods

Reagents

4-NQO and propylene glycol were purchased from Sigma.

Induction of sexual dimorphism of ESCC in mice

All animal procedures were performed in compliance with ethical regulations and approved

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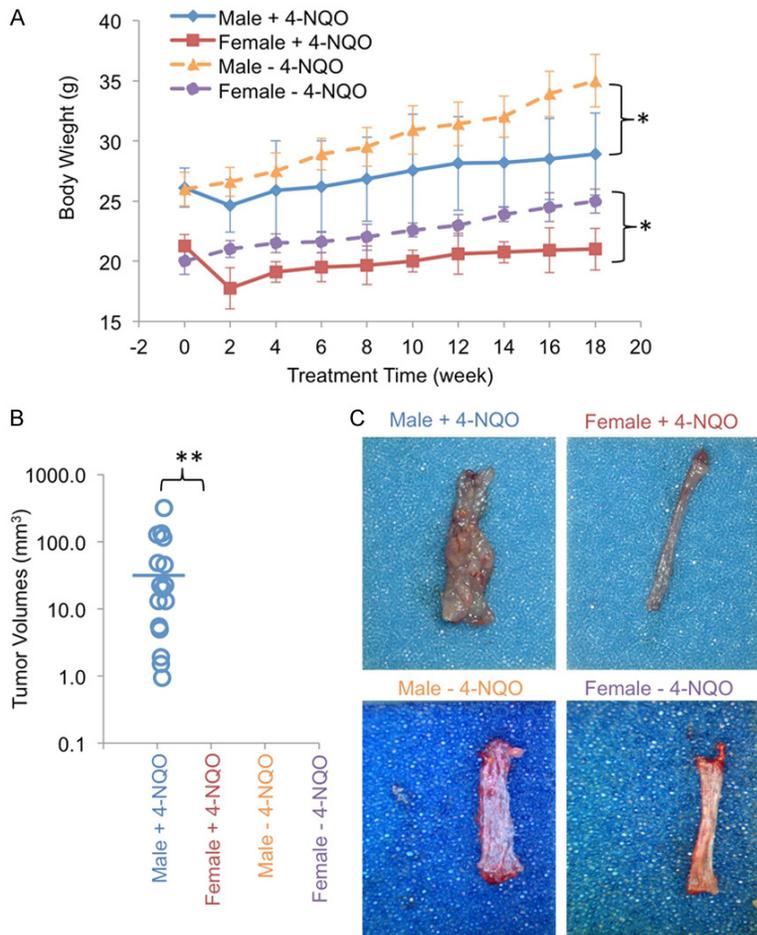


Figure 1. Sex-dimorphic tumorigenesis of ESCC in mice. A. Body weight of male and female mice with (+) and without (-) 4-NQO treatments. *, $P < 0.05$ were found in the comparison between carcinogen-treated and non-treated male or female mice, respectively. B. Tumor volumes of ESCC were measured by small animal ultrasonography. Blue line, mean volume. C. ESCC tumors were induced in male but not in female mice by 4-NQO. No tumors were observed in control mice without 4-NQO treatments. $n = 8$ for each group. **, $P < 0.0001$ was found in the comparison between male and female mice with carcinogen treatment.

by the Institutional Animal Care and Use Committee (IACUC) at the Mayo Clinic. 129J/C57B6L mice were maintained on a normal chow diet. 4-NQO was dissolved in propylene glycol at 5 mg/ml as the stock solution and then diluted in drinking water. Both male and female mice fed with just propylene glycol in drinking water did not induce ESCC as controls [14-17]. Thus, mice (8 males and 8 females) at age of 12 weeks were treated with 18 $\mu\text{g}/\text{ml}$ 4-NQO in drinking water for 8 weeks and mice were then maintained in regular drinking water for 10 weeks. At the end of 18 weeks of tumorigenesis, the entire esophagus was collected for tumor evaluation.

Tumor evaluation

The entire esophagus was opened laterally for tumor evaluation. Tumor volumes and incidences were measured using a small animal ultrasonography (VisualSonic). H&E staining of paraformaldehyde-fixed sections of esophagi were also used for the evaluation of ESCC. Immunohistochemical staining of Ki67 using anti-Ki67 antibodies (Abcam) was used for the evaluation of cell proliferation of ESCC tumors.

Statistical analysis

Data are expressed as mean \pm SEM. One-way ANOVA was used for the analysis of body weight changes between groups during carcinogenesis, tests were 2-tailed, and values of $P < 0.05$ were considered as statistically significant. Fisher's exact test was used for the analysis of tumor incidence and volumes between groups and values of $P < 0.05$ were considered as significant changes.

Results

High concentrations of 4-NQO, such as 100 $\mu\text{g}/\text{ml}$ and 5 mg/ml, have been broadly

used for the induction of ESCC in rodents with 24 to 66 weeks of tumorigenesis periods; however, at these conditions, both male and female rodents developed ESCC, though females developed less tumors than males [14-17]. To identify the cutting point when only males do but females do not develop tumors, we decided to reduce the concentration of 4-NQO and also shorten the latency of carcinogenesis. To develop a clear sex-dimorphic mouse model of ESCC, we used a lower concentration (18 $\mu\text{g}/\text{ml}$) of 4-NQO and a shorter tumorigenesis period of 8 weeks of initiation and 10 weeks of tumor growth. We found that body weights of both male and female mice did not show any clear

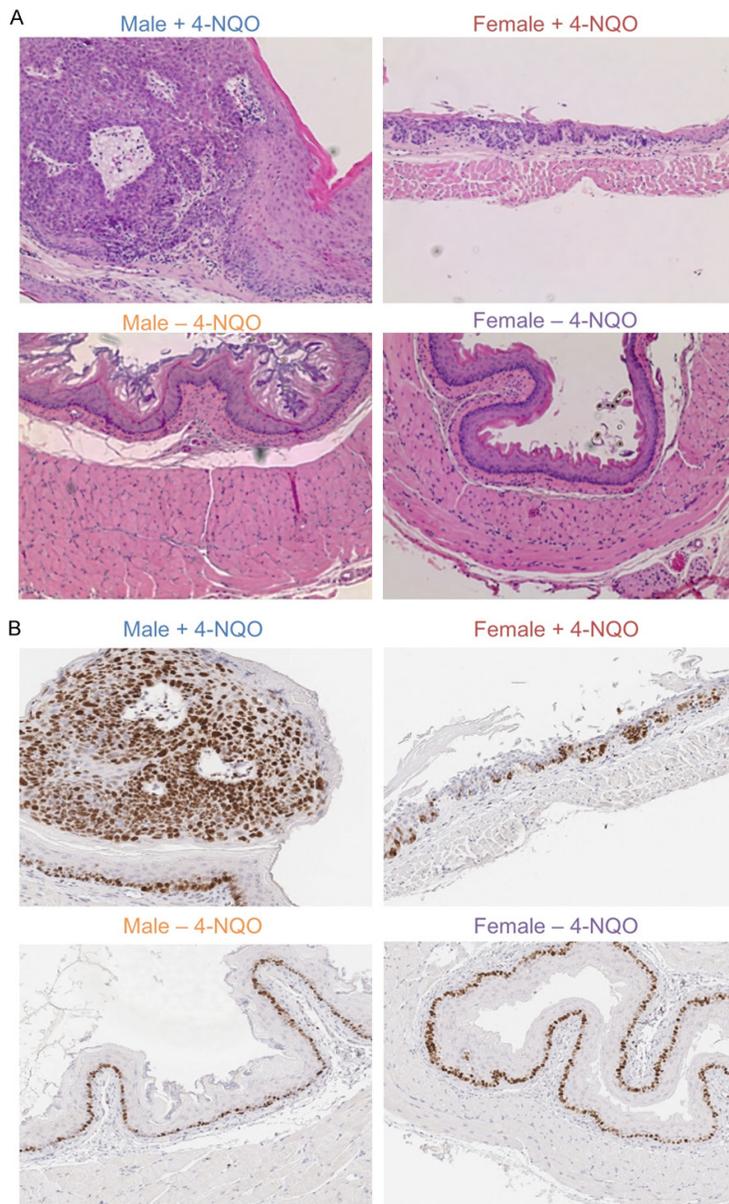


Figure 2. Histological analysis of normal esophagi and ESCC tumors in male and female mice with (+) and without (-) 4-NQO treatments. A. H&E staining (200 x) of male and female esophagi with and without ESCC tumors. B. Immunohistochemical staining (200 x) of Ki67 as the indicator of cell proliferation in male and female esophagi.

changes during 18 weeks of carcinogenesis except for a decrease around 2 weeks after the initial carcinogen treatment, but both male and female mice with carcinogen treatment did show significant reduction in body weights compared to male and female controls without carcinogen treatment, respectively (**Figure 1A**). We found that male mice grew large and/or multiple tumors of ESCC whereas no tumors

were observed in female mice (**Figure 1A**), indicating that male mice are sensitive to the tumorigenesis of ESCC whereas female mice are resistant to it. All male mice had multiple tumors with the volumes of 0.9-316.6 mm³ (**Figure 1B** and **1C**). Next, we performed H&E and Ki67 staining to trace tumor growth and cell proliferation in the esophagi (**Figure 2A** and **2B**). Although we did not observe clear tumors in female mice (**Figure 1B** and **1C**), certain regions of female esophagi showed pre-tumorigenic features, such as increased cell proliferation as indicated by increased nuclei and increased Ki67 staining of epithelial cells (**Figure 2A** and **2B**). Interestingly, esophageal basal epithelial cells were highly positive for Ki67 staining in both male and female mice regardless of carcinogen treatment (**Figure 2B**), indicating potential stem cell-like features of these cells. The 4-NQO treatment induced massive proliferation of ESCC tumor cells in male mice and caused increased proliferation and the loss of lining of epithelial cells in female esophagi (**Figure 2B**). In all, we developed a sex-dimorphic mouse model of ESCC *in vivo* resembling the similar sex-dimorphic incidence of ESCC in humans.

Discussion

Our mouse model with a lower concentration of 4-NQO and a shorter latency of tumorigenesis provides a unique model for investigating sexual dimorphism of ESCC *in vivo*. Addressing the mechanism underlying sexual dimorphism in ESCC or esophageal cancer *in vivo* will help us to fully understand how sexes play the roles in the pathological processes of ESCC tumorigenesis. Our study of developing the sex-dimorphic mouse model of ESCC is prerequisite for better understanding sex-dimorphic incidence of ESCC in humans. Further

studies using this model in combinations with esophagus-specific ablation of sex hormone receptors will provide a clear view on regulatory mechanisms of sex hormone receptors in the sexual dimorphism of ESCC. Given successfully revealing the mechanism of sexual dimorphism in ESCC using our *in vivo* sex-dimorphic model, developing the sex-specific treatments for ESCC patients will be a critical step towards individualized precision medicine. Moreover, future development of sex-dimorphic mouse model for another type of esophageal cancer, esophageal adenocarcinoma, is also imperative for revealing its mechanism of sexual dimorphism. In conclusion, we developed a novel mouse model of esophageal squamous cell carcinoma with clear sexual dimorphism. This model will be beneficial for future *in vivo* studies on sexual dimorphism of esophageal squamous cell carcinoma.

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All authors read the manuscript and consent for publication.

Disclosure of conflict of interest

None.

Abbreviations

ESCC, Esophageal squamous cell carcinoma; 4-NQO, 4-nitroquinoline-1-oxide.

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