Alcohol Promotes Mammary Tumor Development via the Estrogen Pathway in Estrogen Receptor Alpha-Negative HER2/neu Mice

Amy W. Wong, Sarah M. Dunlap, Valerie B. Holcomb, and Nomeli P. Nunez

Background: Alcohol consumption is an established risk factor for breast cancer. Yet, the mechanism by which alcohol affects breast cancer development remains unresolved. The transition from the premenopausal to the postmenopausal phase is associated with a drastic reduction in systemic estrogen levels. It is not clear whether the risk of breast cancer attributable to alcohol consumption is modified by the different levels of estrogen found in pre- and postmenopausal women. The objective of this study is to determine whether the effects of alcohol on mammary tumor development are dependent on the presence of ovarian estrogen.

Methods: As a model of breast cancer, we used mouse mammary tumor virus (MMTV)-neu transgenic mice that overexpress the human epidermal growth factor receptor 2 (HER2/neu) in the mammary epithelium, resulting in the development of estrogen receptor alpha (ERα)-negative mammary tumors. The mammary tumorigenesis process in these mice is similar to that of patients with HER2 breast cancer. Nonovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice were exposed to 0, 5, and 20% ethanol in the drinking water. Breast cancer development and progression were determined alongside the effects of alcohol on estrogen availability and signaling.

Results: Our data show that 20% alcohol consumption promoted tumor development in MMTV-neu mice only in the presence of ovarian hormones. Tumor promotion was associated with increased systemic estrogen levels, increased expression of aromatase (the rate-limiting enzyme in estrogen synthesis), and increased expression of ERα in the tumors of 20% alcohol-consuming MMTV-neu mice. Additionally, we show that ovariectomy (removal of the ovaries and ovarian hormone production) blocked the effects of 20% alcohol on tumor development.

Conclusions: Our results support the notion that alcohol consumption promotes HER2 breast cancer development via the estrogen signaling pathway. Additionally, they suggest that the effects of alcohol on breast cancer may be prevented by blocking estrogen signaling.

Key Words: Alcohol, Breast Cancer, HER2/neu, Estrogen.

APPROXIMATELY 200,000 WOMEN WERE expected to be diagnosed with malignant breast cancer in 2010 and 40,000 will die from the disease (American Cancer Society, 2010). Alcohol consumption is an established risk factor for breast cancer and increases breast cancer risk in a dose-dependent manner: risk increases by ~10% for each drink consumed per day (i.e., risk increases ~20% for 2 daily drinks) (Smith-Warner et al., 1998). A drink is defined as 12 ounces of beer or 5 ounces of wine (Smith-Warner et al., 1998). Not only does alcohol increase the risk of developing breast cancer but it may also increase the risk of breast cancer recurrence and death following breast cancer (Kwan et al., 2010). Several hypotheses have been proposed to explain how alcohol increases breast cancer risk, including (i) alcohol increases breast cancer risk via reactive oxygen species and acetaldehyde (Dumitrescu and Shields, 2005) and (ii) alcohol increases breast cancer risk via hormones such as estrogen and insulin-like growth factor 1 (IGF-1) (Etique et al., 2004). However, none of these hypotheses have been proven. In this study, we examined the role of the estrogen pathway as a mechanism by which alcohol consumption promotes breast cancer using the mouse mammary tumor virus (MMTV)-neu mouse as a model of human epidermal growth factor receptor 2 (HER2) breast cancer.

HER2-positive breast cancers account for 25% of all breast cancer cases (Nahta et al., 2009). Patients with breast cancer over-expressing the HER2 protein (encoded by the neu proto-oncogene also called ErbB2) have poor prognosis (Nahta et al., 2009). In addition, HER2 cancer cells are considered more aggressive (faster growth and invasion) and result in higher rates of reoccurrence in patients (Slamon et al., 1987).

From the Institute for Cell and Molecular Biology (AWW, NPN), University of Texas, Austin, Texas; and Department of Nutritional Sciences (SMD, VBH, NPN), University of Texas, Austin, Texas.

Received for publication May 2, 2011; accepted August 6, 2011.

Reprint requests: Nomeli P. Nunez, PhD, MPH, Division of Nutritional Sciences, One University Station A2703, University of Texas at Austin, Austin, TX 78712; Tel.: 512-471-3243; Fax: 512-471-5844; E-mail: nomeli@mail.utexas.edu

DOI: 10.1111/j.1530-0277.2011.01654.x

To determine the effects of alcohol on HER2 breast cancer, we use MMTV-neu mice. These mice overexpress the neu proto-oncogene in the mammary epithelium, which leads to the development of mammary tumors comparable to those found in patients with HER2 breast cancer (Benz et al., 1992). During the initial stages of tumor development in these mice, tumor progression can be prevented or blocked with the use of tamoxifen, which blocks the binding of estrogen to estrogen receptor alpha (ERα; Menard et al., 2000). However, the use of tamoxifen becomes ineffective in the treatment of these tumors as tumors become estrogen-independent or ERα-negative (Menard et al., 2000). Thus, mammary tumors in MMTV-neu mice are initially estrogen-driven (ERα positive) but become estrogen-independent (ERα negative) as cancer progresses to adenocarcinoma (Menard et al., 2000; Wu et al., 2002).

In women, the transition from the premenopausal to the postmenopausal phase is linked with a drastic reduction in circulating estrogen levels (Ginsburg, 1999; McKinlay, 1996). With respect to alcohol and breast cancer, it is not clear whether the risk of breast cancer attributable to alcohol consumption is altered by the different levels of estrogen found in these 2 phases (Ginsburg, 1999; Mill et al., 2009; Singletary and Gapstur, 2001). In these studies, we determine whether the effects of alcohol on mammary tumor development are affected by the presence/absence of ovarian hormones. For this purpose, we exposed nonovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice to 0, 5, and 20% alcohol in the drinking water and assessed the effects of the different doses of alcohol on mammary tumor development and progression.

Results show that 20% alcohol promotes mammary tumor development in MMTV-neu mice but only in the presence of ovarian estrogens; ovariectomy, resulting in the absence of ovarian estrogen, blocked the effects of 20% alcohol on tumor development in MMTV-neu mice. The acceleration of tumor development as a result of alcohol consumption is associated with increased systemic estrogen levels, increased tumor expression of aromatase, and increased expression of the ERα in tumor tissues. Our data support the hypothesis that alcohol consumption promotes mammary tumor development via the estrogen pathway.

MATERIALS AND METHODS

Mouse Husbandry and Diets

A total of 180 pathogen-free female MMTV-neu mice in the FVB/N genetic background were purchased from The Jackson Laboratory (Bar Harbor, MA). These mice were obtained at 6 weeks of age and housed in the Animal Resources Center at the University of Texas at Austin (UT-Austin) in accordance with NIH guidelines. All animal procedures were approved by UT-Austin's Institutional Animal Care and Use Committee. Mice were singly housed in a 22 to 24°C room and kept on a 12-hour light/dark cycle. Following an acclimation period of 1 week, mice were randomized into 6 groups: 90 NOVX mice and 90 OVX mice; each of these groups was divided into 3 subgroups: 30 mice not consuming alcohol (control groups), 30 mice consuming 5% v/v ethanol, and 30 mice consuming 20% v/v ethanol ad libitum throughout the study. Alcohol treatment began at 9 weeks of age. Mice were fed Diet #D12450B (Research Diets Inc., New Brunswick, NJ), composed of 19% protein, 67% carbohydrate, and 4% fat, ad libitum throughout the study. Body weight, food and liquid consumption, and tumor volume were measured weekly. Serum was collected at the endpoint (week 52) for analysis.

Body Composition

Final body weight was determined at the end of the study. All mice were used for final body weight analysis ($n = 30$ per group). Percentage body fat was determined using dual-energy X-ray absorptiometry with a GE Lunar Piximus II densitometer (Waukesha, WI). A total of 8 mice per group were used for percentage body fat analysis ($n = 8$ per group).

Ovariectomy

Mice in the OVX groups were OVX at 8 weeks of age. Mice were anesthetized and a small midline incision (~1.0 cm) was made in the skin halfway between the middle of the back and the base of the tail, starting at the last rib. A small incision was then made through the peritoneal lining on each side, and the ovaries were removed with a single cut between the fallopian tube and the uterine horn.

Tumor Development

To detect the appearance of tumors, mice were palpated weekly starting at the age of 13 weeks until the first tumors appeared. Following the appearance of the first tumor, mice were palpated twice weekly, and tumor volume was determined by measuring the length, width, and depth of the tumor using Fisherbrand digital calipers (Thermo Fisher Scientific, Rockford, IL). Mice bearing tumors were sacrificed either at 52 weeks or once tumors reached 1.5 cm^3, whichever occurred first. At the end of the 52-week study, tumors were collected and fixed in 10% buffered formalin and embedded in paraffin. The z-test for proportions statistical analysis was used to determine significance in tumor incidence. All mice were used for tumor incidence analysis ($n = 30$ per group). Tumor growth rate was determined by measuring the difference in tumor volume per day. All mice bearing tumors were used for tumor growth rate analysis ($n \geq 3$ per group).

Serum Analysis

At necropsy, blood from mice was collected via cardiac puncture, allowed to clot for 20 minutes, and centrifuged at 6,000×g for 5 minutes. Serum was separated and stored at −80°C until analysis. Serum estrogen levels were measured using an ELISA for 17β-estradiol (E2; IBL-America, Minneapolis, MN). Each sample was assayed in duplicate on a single plate. A total of 8 mice per group were used for analysis ($n = 8$ per group). The reported range of this assay is 9.7 to 2,000 pg/ml. Blood alcohol levels were determined by measuring alcohol dehydrogenase activity using the Sigma-Aldrich NADH assay (St. Louis, MO). A total of 9 mice per group were used for this analysis ($n = 9$ per group).

Immunohistochemistry

Mouse tumor tissues were harvested and fixed in 10% formalin for 24 hours before transferring to 70% ethanol. Samples were paraffin embedded, sectioned to obtain unstained tissue slides, and stained to detect c-erbB2 (sc-284, 1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA), Ki67 (#M7249, 1:200; Dako, Glostrup, Denmark), and ERα (sc-542, 1:500; Santa Cruz Biotechnology Inc.) by the Histology and Tissue Core at the University of Texas M. D. Anderson Cancer

Center (Smithville, TX). Results were quantitated using NIS Elements Imaging Software (Nikon Precision Inc., Belmont, CA). A total of 4 tissue samples per group were used for analysis for each protein ($n = 4$ per group).

Whole Mounts

Mouse mammary gland #9 was harvested and fixed onto slides with 10% formalin for 24 hours before transferring to 70% ethanol. Samples were then processed by the Histology and Tissue Core at the University of Texas M. D. Anderson Cancer Center. Visual examination of the mammary whole mounts was carried out by pathologists at the University of Texas. No quantitation was performed.

Immunoblot

Mouse tumor tissues were harvested at necropsy, snap-frozen in liquid nitrogen, and stored at −80°C until analysis. Whole-cell lysates were prepared from tumor tissue. Frozen tissues were ground into powder and suspended in RIPA buffer (Sigma) containing protease and phosphatase inhibitors (Pierce, Rockford, IL). Samples were then homogenized, and extracts were clarified by centrifugation at 6,000×*g* for 10 minutes. A Bradford protein assay (Bio-Rad, Hercules, CA) was used to determine protein concentrations. Lysates (50 μg) were resolved by 10% SDS-PAGE, and proteins were detected by immunoblot. Expression of tumor aromatase, ERα, p-MAPK, MAPK, p-Akt, and Akt were analyzed by immunoblotting using antibodies for the following proteins: aromatase (ab18995, 1:1,000; Abcam, Cambridge, MA), ERα (sc-542, 1:200; Santa Cruz Biotechnology Inc.), p-MAPK (#9101, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA), MAPK (#9102, 1:1,000; Cell Signaling Technology, Inc.), p-Akt (#9275, 1:1,000; Cell Signaling Technology, Inc.), and Akt (#9272, 1:1,000; Cell Signaling Technology, Inc.) followed by incubation with an anti-rabbit secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc.) and ECL substrate (Thermo Fisher Scientific). Quantitation of the band intensity was determined by densitometry using ImageJ software (NIH website: http://rsbweb.nih.gov/ij/index.html). A total of 3 tumor tissue samples were used per group ($n = 3$) for analysis.

Statistical Analysis

Data are expressed as means ± SEM. Significant differences among control and treatment groups were determined using 2-way analysis of variance followed by Bonferroni adjustment unless otherwise noted. SPSS v16 for Windows (IBM Corporation, Chicago, IL) was used for all statistical comparisons. To detect statistical significance, *p*-value was set to 0.05.

RESULTS

Body Weight and Body Fat

High body weight and high body fat levels are risk factors for breast cancer (Key et al., 2003). Results show that the body weight and body fat levels in alcohol and non-alcohol-consuming mice were similar (Fig. 1*A*,*B*, $p > 0.05$). The only noticeable effect was that of ovariectomy, which increased the susceptibility of gaining body weight and body fat levels (Fig. 1*B*). Although ovariectomy resulted in an increase in both body weight and body fat levels, interestingly, these effects did not translate to increased tumor incidence or growth (Figs. 1 and 3). Thus, results suggest that alcohol does not affect tumor development via body weight or body fat levels.

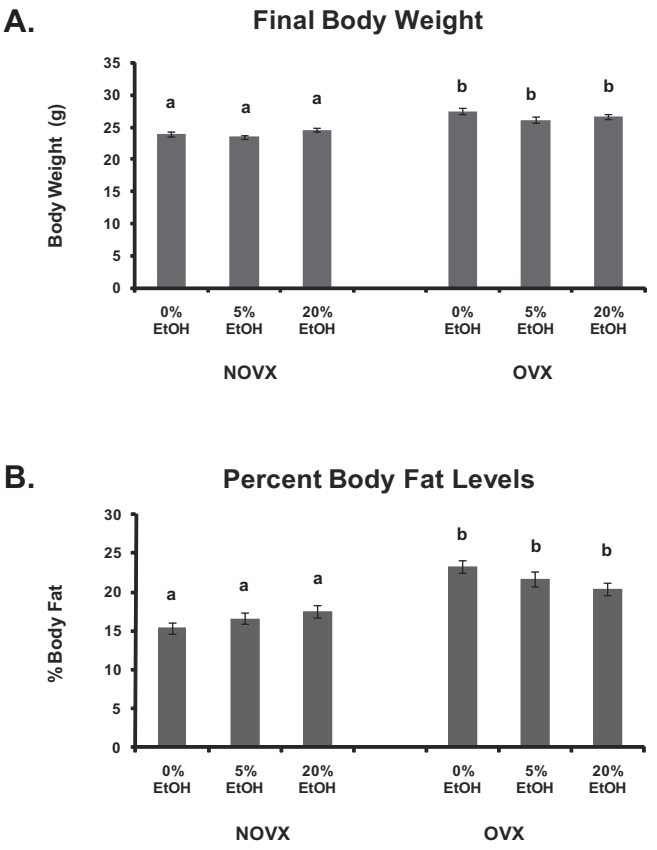

Fig. 1. Final body weight and percentage body fat in mouse mammary tumor virus (MMTV)-neu mice. (**A**) Body weight of nonovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice after 52 weeks of alcohol exposure. The results are expressed as the mean ± SEM of 30 mice per group ($n = 30$ per group). (**B**) Percentage body fat levels of NOVX and OVX MMTV-neu mice as determined by dual-energy X-ray absorptiometry following 52 weeks of alcohol exposure. The results are expressed as the mean ± SEM of 8 mice per group ($n = 8$ per group). For each graph, different lowercase letters represent statistical significance ($p < 0.05$). EtOH, ethanol.

Blood Alcohol Content in MMTV-neu Mice

Consumption of 5 and 20% v/v ethanol led to low (∼0.01%) and medium high (∼0.05%) blood alcohol levels, respectively, in the mice (Fig. 2). At present, there is limited knowledge in literature describing the relationship between blood alcohol levels, drinks/day, and breast cancer risk and linking this information from animal to human studies. From what we can currently extrapolate from literature, consumption of 1 to 2 drinks and 3 to 4 drinks in women can lead to similar (∼0.02 and ∼0.05%, respectively) blood alcohol levels, depending on body weight (State of Iowa Alcoholic Beverages Division, 2011). According to a study that evaluated data from over 300,000 women for up to 11 years, consumption of alcohol at these levels significantly increased breast cancer risk approximately 16 and 41%, respectively (Smith-Warner et al., 1998).

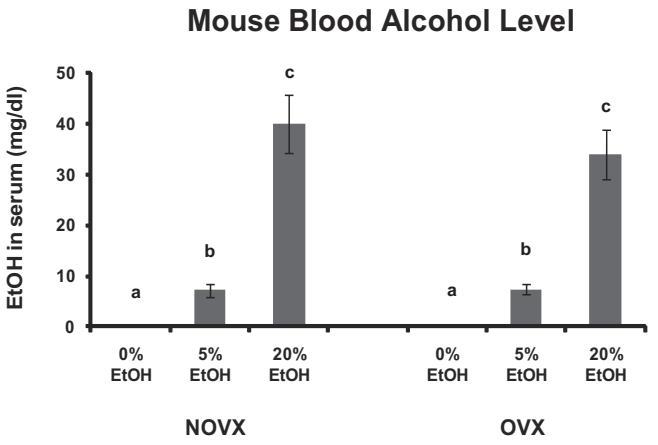

Fig. 2. Blood alcohol content in mouse mammary tumor virus-neu mice. Blood alcohol levels were determined in all groups of mice by assaying alcohol dehydrogenase activity in mouse sera. The results are expressed as the mean ± SEM of 9 mice per group ($n = 9$ per group). Different lowercase letters represent statistical significance ($p < 0.05$). EtOH, ethanol; OVX, ovariectomized; NOVX, nonovariectomized.

Alcohol Promotes Tumor Development in MMTV-neu Mice

Results show that alcohol consumption increased tumor incidence in a dose-dependent manner in NOVX mice but not in OVX mice (Fig. 3*A*). Tumor incidence was defined as the appearance of a single palpable tumor on an animal. (In this study, multiple tumors on the mammary gland of a single animal were not observed.) Tumor incidence in the 5 and 20% alcohol-consuming NOVX mice was 53.33 and 66.67%, respectively, compared to 40 in 0% alcohol-consuming mice, at the endpoint of study (Fig. 3*A*). Among NOVX mice, statistical significance in tumor incidence was seen only between the 0 and 20% alcohol-consuming mice ($p < 0.05$). In addition, alcohol decreased the tumor latency period in NOVX mice (Fig. 3*A*). Conversely, no statistically significant change in mammary tumor incidence or tumor latency was observed among the OVX groups as a result of alcohol consumption ($p > 0.05$). Thus, it is likely that alcohol promotes mammary tumor development only in the presence of normal systemic estrogen levels, which the OVX animals lack because of the surgical removal of the ovaries.

Results also show that 20% ethanol treatment led to a significant increase in tumor growth rate ($p < 0.05$) in NOVX mice (Fig. 3*B*). Tumor growth rate was determined using the following equation: [(Final tumor volume)−(Tumor volume at first detection)]/(Number of days tumor was present). This suggested that alcohol may promote cancer cell proliferation in NOVX mice. To determine whether this was the case, protein levels of the proliferation marker Ki67 in tumor tissues were measured (Schlüter et al., 1993). Findings show alcohol significantly increased the level of Ki67 in the tumors of NOVX mice consuming 20% alcohol (2.6-fold change, $p < 0.05$) but not in any other groups of mice (Fig. 3*C*).

Alcohol Consumption Affects the Mammary Gland Structure

Mammary gland morphology may be used as a tool for assessing breast cancer risk in women (Brisson et al., 1989; Byrne et al., 1995; Pasqualin, 2002). High mammographic density, which is associated with a high number of terminal ductal lobular units (TDLUs), can increase the risk of breast cancer 4- to 6-fold (Pasqualin, 2002). In the mouse, the TDLU is more often referred to as the lobualveolar (LA) structure (Cardiff, 1998). We examined the effects of alcohol on the structure of the mammary gland of alcohol-consuming mice. Figure 4 shows that alcohol consumption increased the number of LA (TDLU in humans), which is the site where many epithelial hyperplasias and carcinomas of the breast arise (Byrne et al., 1995; Cardiff, 1998; Pasqualin, 2002; Stolier and Wang, 2008). This suggests that alcohol may increase the number of cells that can potentially become cancer cells in the mammary gland.

Effects of Alcohol on Systemic Estrogen and Tumor Aromatase Levels

Alcohol may exert its effects on breast cancer by increasing estrogen (E2) availability (Dorgan et al., 2001; Ginsburg et al., 1996; Zhang et al., 2007). To determine whether alcohol affected systemic estrogen levels, we measured the levels of E2 in the serum of our mice. Results in Fig. 5*A* shows that 20% alcohol significantly increased E2 levels in NOVX mice compared to NOVX mice consuming 0 and 5% alcohol ($p < 0.05$). Moreover, results show ovariectomy decreased systemic E2 levels in OVX mice ($p < 0.05$) to levels below the sensitivity of the ELISA, thereby verifying that the ovariectomy surgeries were successful. While 20% alcohol consumption increased estrogen levels in OVX mice, the estrogen levels were still significantly lower than those of NOVX control mice. As OVX mice lack ovaries, any estrogen found in their blood may come from peripheral tissues such as adipose or tumor tissue (Reed and Purohit, 1997).

The rate-limiting enzyme in the synthesis of estrogen in these tissues is known as cytochrome p450 aromatase (Brodie et al., 2001; Reed and Purohit, 1997). Alcohol may increase systemic estrogen levels by affecting the expression of aromatase in tissues (Brodie et al., 2001; Purohit, 2000). Thus, we measured aromatase levels in the tumor tissues of the NOVX and OVX animals to determine whether alcohol affected the expression of this enzyme. We found that the pattern of aromatase expression was similar to systemic E2, with NOVX mice expressing higher levels in tumor tissues compared to OVX mice (Fig. 5*A*,*B*, $p < 0.05$). In addition, Fig. 5*B* shows 20% alcohol consumption in MMTV-neu mice resulted in higher tumor aromatase expression compared to 0% alcohol-consuming mice in both NOVX and OVX groups ($p < 0.05$). Thus, our data show that the tumor-promoting effects of alcohol on NOVX mice are associated with an increase in systemic estrogen levels and an increase in tumor aromatase expression.

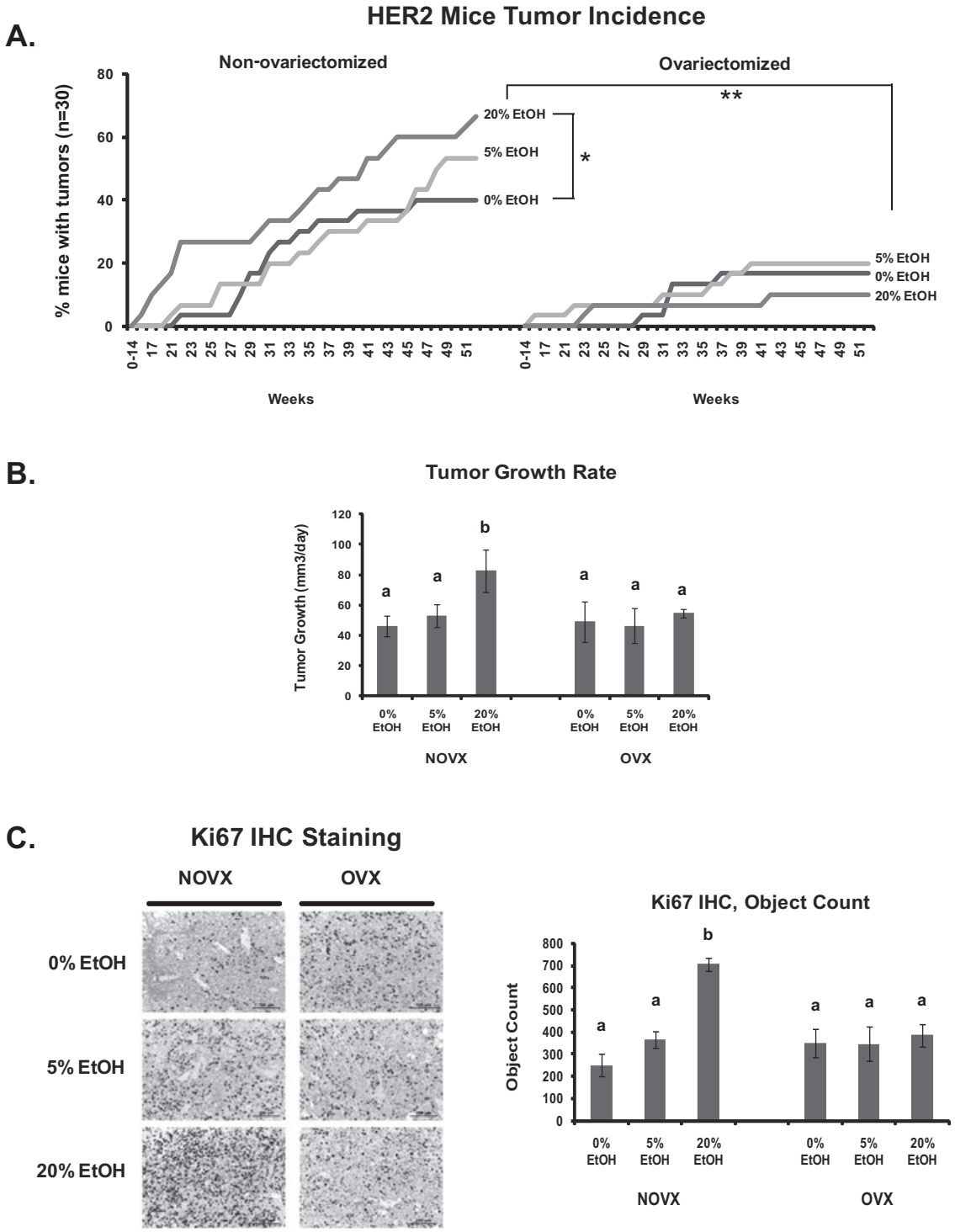

Fig. 3. Effects of alcohol on HER2 tumor development and progression in mouse mammary tumor virus (MMTV)-neu mice. (**A**) Alcohol consumption of 5 and 20% v/v ethanol increased mammary tumor incidence of nonovariectomized (NOVX) MMTV-neu mice but not ovariectomized (OVX) mice. Tumor incidence was determined by palpating mice at least once per week throughout the study. The results represent the percentage of mice ($n = 30$ per group) exhibiting palpable tumors. The *z*-test for proportions was used to determine statistical significance. Asterisks represent significance ($p < 0.05$). *Significance between 0 and 20% alcohol-consuming NOVX mice. **Significance between NOVX and OVX mice on their respective liquid diet groups (e.g., 0% EtOH NOVX vs. 0% EtOH OVX, 5% EtOH NOVX vs. 5% EtOH OVX, etc.). (**B**) Consumption of 20% v/v ethanol significantly increased tumor growth rate within NOVX MMTV-neu mice but not OVX mice. Tumor growth rate was determined by: [(Final tumor volume)−(Tumor volume at first detection)]/(Number of days tumor was present). The results are expressed as the mean ± SEM of all tumor-bearing mice per group ($n \geq 3$ per group). Different lowercase letters represent statistical significance ($p < 0.05$). (**C**) Consumption of 20% v/v ethanol significantly increased cellular proliferation in NOVX MMTV-neu mice but not OVX mice. Proliferation was determined by immunohistochemical (IHC) staining of cellular proliferation marker Ki67. Quantitation of results using imaging software is shown alongside a representative of stained slides. The results are expressed as the mean ± SEM of 4 quantitated slides per group ($n = 4$ per group). Different lowercase letters represent statistical significance ($p < 0.05$). EtOH, ethanol; HER2, human epidermal growth factor receptor 2.

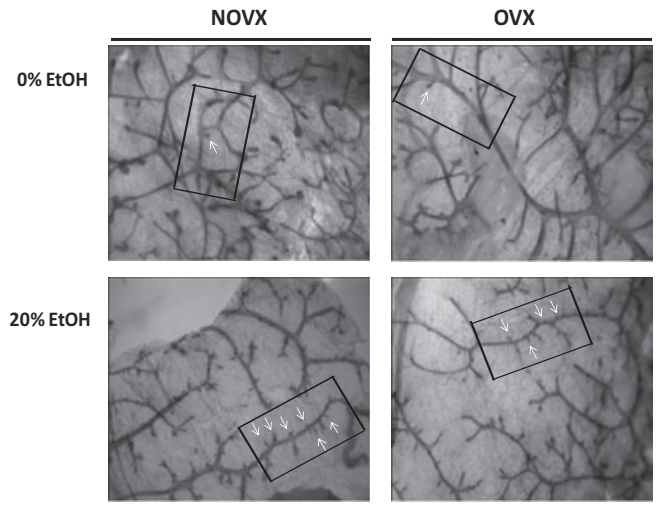

Fig. 4. Effects of alcohol on the structure of the mammary gland. Alcohol consumption alters mammary gland morphology in nonovariectomized (NOVX) and ovariectomized (OVX) mouse mammary tumor virus-neu mice. Whole mounts were obtained from mouse mammary gland #9. Most pronounced is the effect of 20% v/v ethanol consumption on mammary gland structure compared to nonalcohol groups, as can be seen by the increased lobualveolar structures (indicated by arrows). The images above are representatives of whole mounts taken from at least 3 slides per group ($n \geq 3$ per group). EtOH, ethanol.

Alcohol Increases Expression of ERα in MMTV-neu Mice

Estrogen stimulates tumor growth and proliferation of breast cancer cells by binding to the receptor ERα (Etique et al., 2006). Tumor ERα expression in alcohol and nonalcohol-consuming mice was measured to determine whether the tumor-promoting effects of alcohol were associated with increased ERα expression. Immunohistochemical (IHC) results show that alcohol increased the expression of ERα in both NOVX and OVX tumors in MMTV-neu mice that are reported to develop ERα-negative tumors (Fig. 6*A*; Li et al., 2007). These results were verified by immunoblot with breast cancer cell lines MCF-7 and MDA-MB-231 as the positive and negative controls, respectively, for ERα expression (Fig. 6*B*). Although alcohol consumption increased the expression of ERα in OVX mice, this increase in ERα by alcohol was not associated with an increase in tumor incidence, tumor growth, or cellular proliferation in the OVX 5 or 20% alcohol-consuming mice, suggesting that ERα expression by itself is not sufficient to promote tumor development. Thus, it is feasible that alcohol-mediated HER2 breast cancer development requires the presence of normal circulating estrogen levels, which OVX animals lacked.

Alcohol Activates the Estrogen Signaling Pathway

The aforementioned data suggest alcohol may promote HER2 tumor development through the estrogen pathway. Thus, we determined the activation of MAPK and Akt, 2 downstream targets of the estrogen signaling cascade, which may signify the activation of the estrogen pathway (Peng and Jordan, 2008). Expression of total MAPK and Akt, and their active, phosphorylated forms, p-MAPK and p-Akt, were determined by immunoblot using tumor tissues. Consumption of 5 and 20% alcohol significantly increased the activation of these downstream targets in the estrogen pathway in NOVX MMTV-neu mice ($p < 0.05$) but not in OVX mice ($p > 0.05$), as shown in Fig. 7.

Alcohol Does Not Affect erbB2 Transgene Expression or IGF Receptor Levels

To verify that alcohol did not affect tumor development by increasing the expression of the erbB2/HER2 transgene, we measured HER2 levels in the tumors by immunohistochemistry. HER2 levels were similar among all the groups consuming alcohol and nonalcohol (data not shown). Moreover, ovariectomy did not affect HER2 levels (data not shown). We also measured systemic levels of IGF-1, a hormone that may promote mammary tumor development (Peyrat et al., 1993); systemic IGF-1 levels were not affected by alcohol consumption (data not shown). In addition, we measured the expression of the IGF-1 receptor in the tumors and found the levels similar among all groups (data not shown) (Werner and Bruchim, 2009). Thus, these findings suggest that alcohol does not affect mammary tumor development by affecting the expression of the HER2 transgene or by affecting the IGF-1 signaling pathway.

DISCUSSION

Previous studies have reported an association between alcohol abuse or excessive drinking (≥3 drinks/day) and an increase in mammary tumor incidence in women (studies summarized by Singletary and Gapstur, 2001). Smith-Warner and colleagues (1998) also reported a linear relationship between alcohol and breast cancer, for ethanol intake of up to 60 grams per day (approximately 4.5 bottles of beer, 5.6 glasses of wine, or 4 shots of liquor). However, the mechanism by which alcohol consumption contributes to mammary carcinogenesis remains unresolved. Our studies provide direct evidence suggesting alcohol increases mammary tumor development via the estrogen pathway. Our data show that alcohol consumption promotes HER2 mammary tumor development in MMTV-neu mice but only in the presence of ovarian hormones. The acceleration of tumor development as a result of alcohol consumption is associated with increased systemic estrogen levels and increased expression of aromatase and ERα in tumor tissues. Furthermore, we show that the effects of alcohol on tumor development in HER2 mice can be blocked by ovariectomy. As alcohol only promoted tumor development in the presence of physiologically normal systemic levels of estrogen, results suggest that the effects of alcohol on HER2 mammary tumor development might be mediated via estrogen. However, one caveat in this study is that OVX mice failed to develop tumors in numbers comparable to NOVX mice. It is therefore important to keep in mind that the presence of steroidal hormones such as estrogen

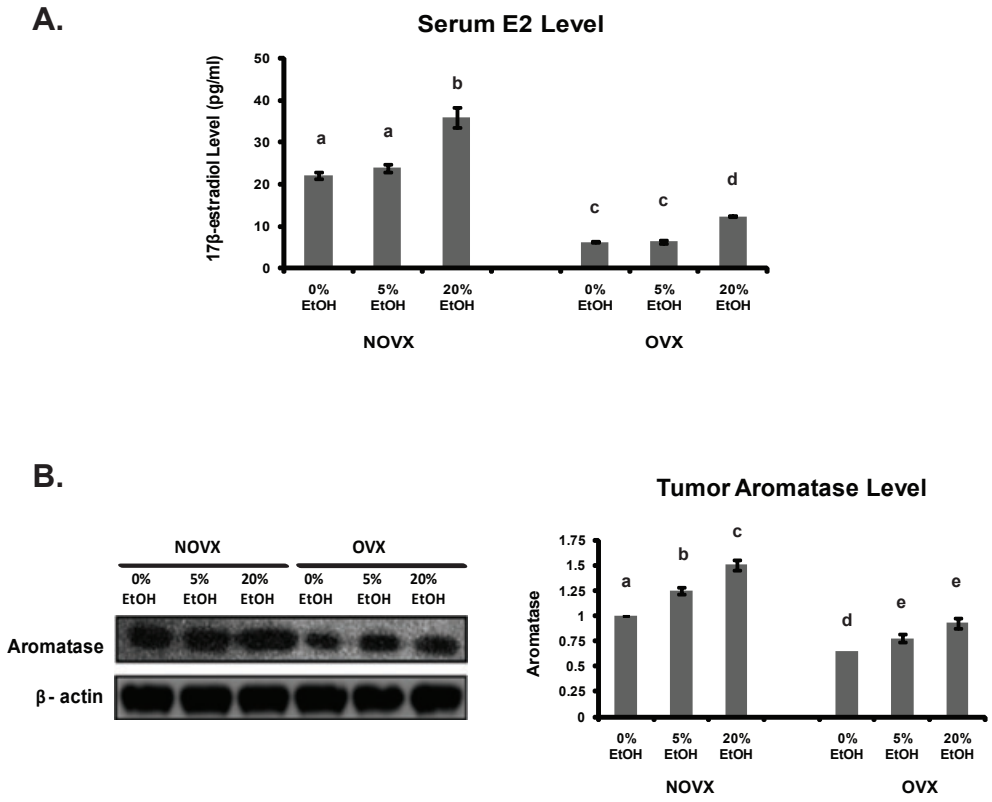

Fig. 5. Alcohol promotes estrogen availability in mouse mammary tumor virus (MMTV)-neu mice. (**A**) Systemic 17β-estradiol (E2) levels were significantly increased in nonovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice consuming 20% v/v ethanol compared to their 0 and 5% alcohol-consuming counterparts at 52 weeks following initial alcohol exposure. E2 levels were determined by ELISA. (Note: OVX mice consuming 0 and 5% v/v ethanol exhibited average estradiol levels, which fell below the sensitivity level of the ELISA and may be considered as nondetectable.) The results are expressed as the mean ± SEM of 8 mice per group (n = 8 per group). Different lowercase letters represent statistical significance ($p < 0.05$). (**B**) Tumor aromatase expression was significantly increased in alcohol-consuming NOVX and OVX mice compared to non-alcohol-consuming groups. Aromatase levels were determined by immunoblot. Bands migrated at approximately 55 kDa. Quantitation of results by densitometry is shown alongside a representative immunoblot. The results are expressed as the mean ± SEM of 3 quantitated blots per group (n = 3 per group). Different lowercase letters represent statistical significance ($p < 0.05$). EtOH, ethanol.

may be important for the tumor model in general and that failure to see tumor promotion with alcohol is a secondary effect.

Nevertheless, existing evidence suggests alcohol may affect breast cancer via the estrogen pathway (Chen et al., 2002; Dorgan et al., 2001; Etique et al., 2004; Ginsburg et al., 1996; Zhang et al., 2007) and elevated estrogen levels are considered a risk factor for breast cancer (Chen et al., 2002), which our data support. Epidemiological studies show that women who consume alcohol have higher systemic estrogen levels (Chen et al., 2002; Chung, 1990; Gapstur et al., 1992). It is possible that alcohol increases systemic estrogen levels by increasing the level of aromatase, an enzyme that converts androgens to estrogens (Chen et al., 2002; Chung, 1990; Etique et al., 2006; Gapstur et al., 1992). According to Gordan and colleagues (1979), as aromatization involves a series of hydroxylation reactions, it is not unexpected that alcohol, a known hydroxylase inducer, increases the level of activity of the aromatase enzyme. High levels of estrogen may promote the development of breast cancer by activating the estrogen pathway to stimulate cellular proliferation (Tan et al., 2009). In addition, previous studies using cell culture conditions show that alcohol exposure increases the expression of ERα in cancer cells (Fan et al., 2000). Thus, not only does alcohol increase systemic estrogen levels but it also increases the expression of its receptor ERα, which may explain how breast cancer cells are more sensitive to estrogen in the presence of alcohol (Chen et al., 2002; Chung, 1990; Gapstur et al., 1992). This may suggest that alcohol consumption in conjunction with estrogen replacement therapy may increase breast cancer risk to a higher degree than either treatment alone. In fact, alcohol in conjunction with estrogen-containing hormone replacement therapy (HRT) increases breast cancer risk to a greater extent than HRT or alcohol alone (Chen et al., 2002). For example, alcohol alone increases breast cancer risk by 28%, estrogen alone by 45%; however, the combination of estrogen and alcohol elevates the risk by 108% (Chen et al., 2002). Although studies show alcohol exposure increases systemic estrogen levels in women, and in cell culture conditions alcohol increases the expression levels of aromatase and ERα, it is not known how the effects of alcohol in the estrogen signaling pathway relate to breast cancer development (e.g., tumor

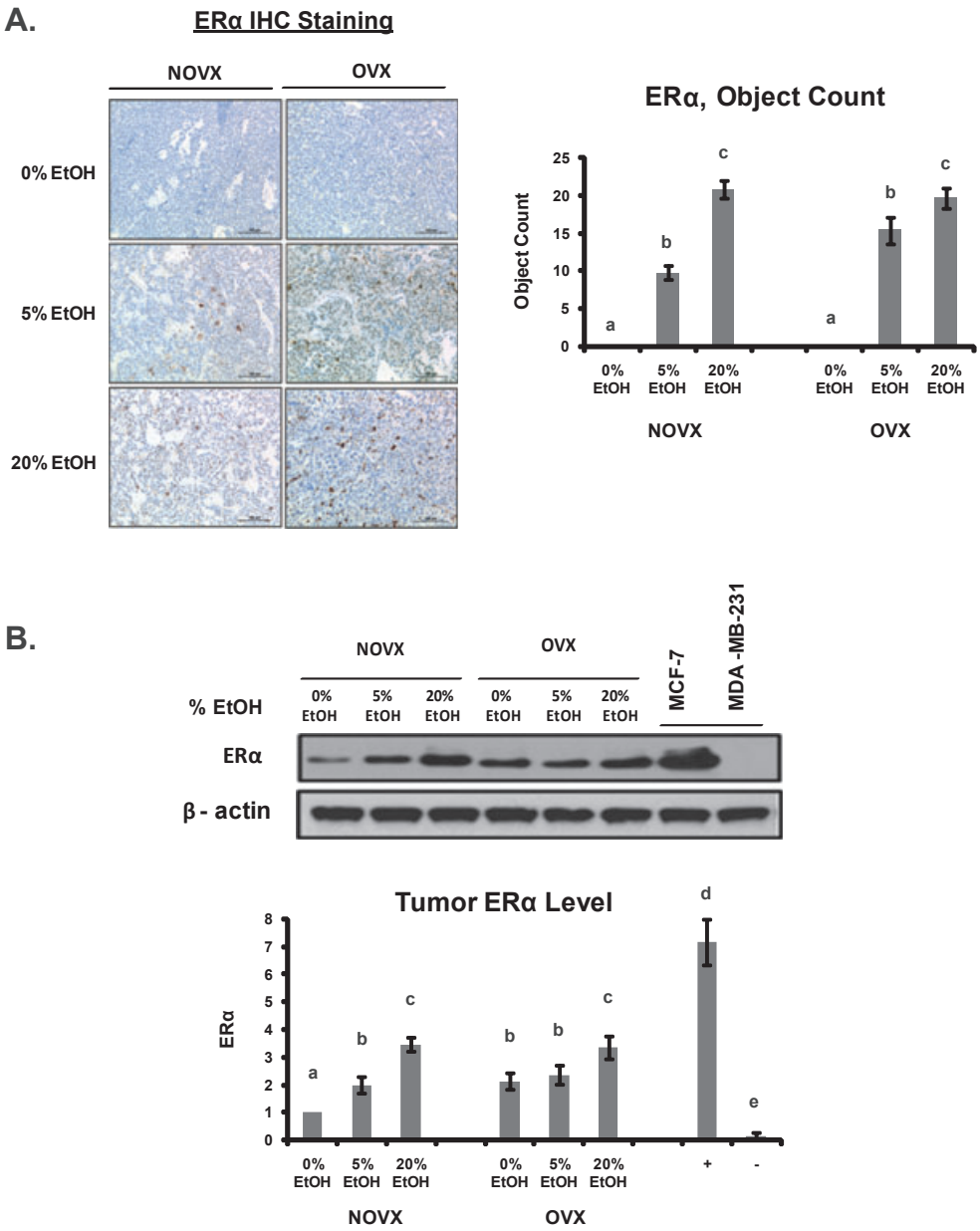

Fig. 6. Alcohol promotes the expression of estrogen receptor alpha (ERα) in mouse mammary tumor virus (MMTV)-neu mice. Tumor ERα expression levels were significantly increased in nonovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice consuming alcohol compared to the nonalcohol groups following 52 weeks of alcohol exposure. Tumor ERα expression was determined by (**A**) immunohistochemical (IHC) staining and (**B**) immunoblot. Quantitation of IHC results using imaging software is shown alongside a representative of stained slides. The results are expressed as the mean ± SEM of 4 quantitated slides per group ($n = 4$ per group). Quantitation of immunoblots by densitometry is shown alongside a representative immunoblot. MCF-7 and MDA-MB-231 breast cancer cells were used as positive and negative controls, respectively, for ERα expression. The results are expressed as the mean ± SEM of 3 quantitated blots per group ($n = 3$ per group). For each graph, different lowercase letters represent statistical significance ($p < 0.05$). EtOH, ethanol.

incidence, tumor growth). Results of this study support the role of the estrogen pathway as a mechanism by which alcohol consumption promotes the development of HER2 mammary cancer.

The MMTV-neu mouse model of HER2 breast cancer overexpress the wild-type erbB2 gene in the mammary gland and develop ERα-negative mammary carcinomas (Guy et al., 1992; Li et al., 2007). Initially, the tumors are estrogen-driven (ERα positive) but become estrogen-independent as cancer progresses to adenocarcinoma. The course of mammary tumorigenesis in these mice is similar to that of humans, which proceeds from hyperplasia, to ductal carcinoma in situ, to invasive breast cancer (Li et al., 2007). Interestingly, alcohol consumption increased mammary tumor incidence and growth, decreased tumor latency, and led to the development of ERα-positive tumors in MMTV-neu mice. The expression of ERα in the tumor tissues suggest that alcohol may either (i) prevent the loss or extend the expression and function of ERα, or (ii) cause reexpression of ERα, in an otherwise ERα-negative breast cancer subtype.

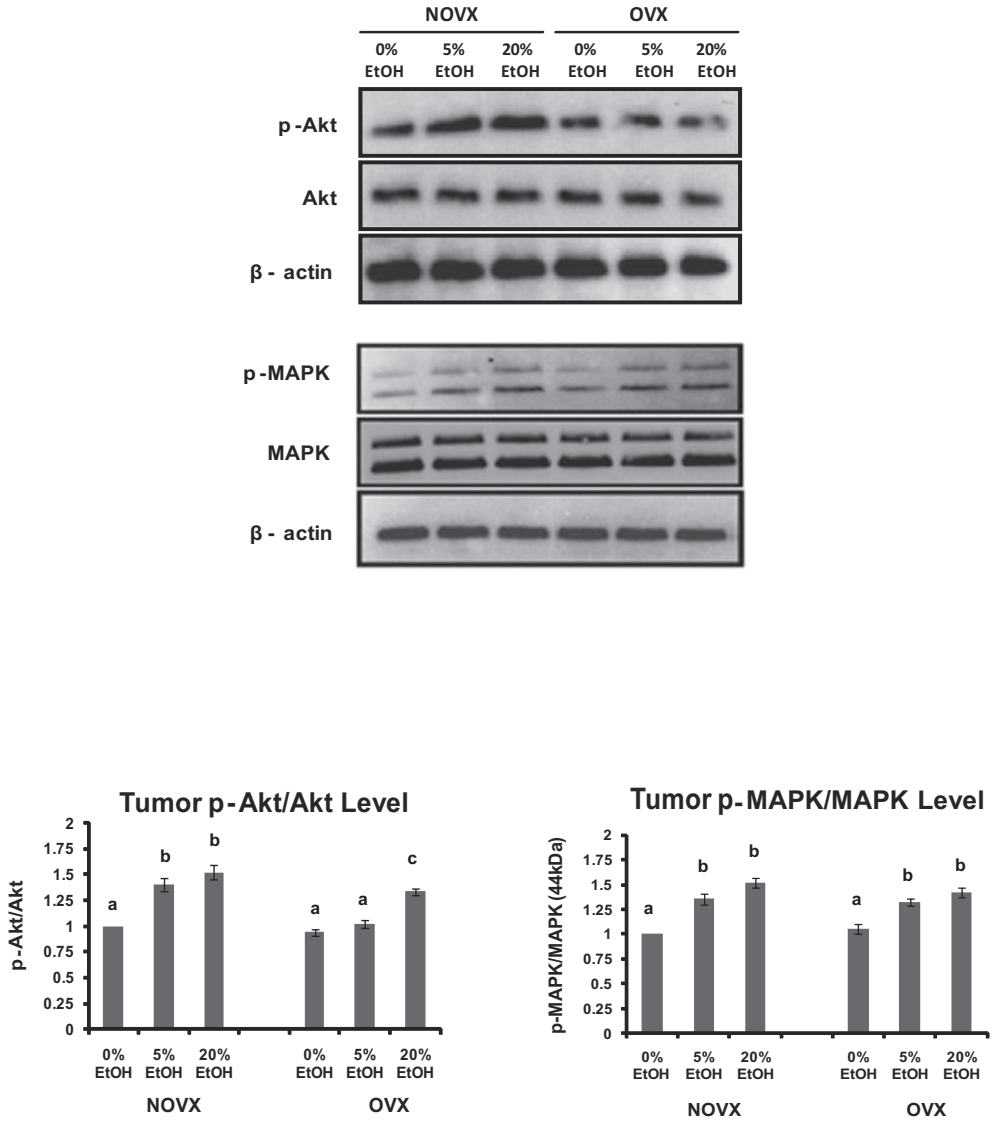

Fig. 7. Alcohol consumption in nonovariectomized (NOVX) mice activates downstream targets of the estrogen signaling pathway. Alcohol consumption increased the activation of Akt and MAPK, downstream targets of the estrogen signaling pathway, in NOVX but not ovariectomized (OVX) mouse mammary tumor virus-neu mice. Expression of Akt, p-Akt, MAPK and p-MAPK in tumor tissues were determined by immunoblot. Quantitation of results by densitometry is shown alongside a representative immunoblot. The results are expressed as the mean ± SEM of 3 quantitated blots per group ($n = 3$ per group). Different lowercase letters represent statistical significance ($p < 0.05$). EtOH, ethanol.

Evidence suggests that the loss of ERα gene expression in ERα-negative breast cancers is not the result of DNA mutations in the ERα gene (Li et al., 2010; Roodi et al., 1995). Therefore, it is possible that the loss of ERα expression may be due to decreased transcription. In fact, previous studies show that aberrant methylation status of the ERα promoter appears in more than 25% of ERα-negative breast cancer cells (Lapidus et al., 1998; Ottaviano et al., 1994; Yang et al., 2001). Histone acetylation/deacetylation has also been implicated as a possible mechanism by which ERα transcription is repressed (Yang et al., 2000). Thus, epigenetic regulation of ERα may play an important role in the loss of ERα expression in ERα-negative breast cancer subtypes such as the HER2 subtype. Our objective in future studies will be to determine the mechanism by which alcohol affects the expression of ERα gene.

With respect to systemic estrogen levels, numerous studies have shown that alcohol consumption increases systemic estrogen levels in both pre- and postmenopausal women (Chen et al., 2002; Chung, 1990; Gapstur et al., 1992). In our own studies, we show that alcohol increased systemic estrogen levels in both NOVX and OVX female mice. Even though alcohol increased estrogen levels in OVX mice, the levels were still lower than those of NOVX mice not consuming alcohol. As OVX mice lack ovaries, the estrogen found in their blood may originate from peripheral tissues (i.e., adipose or tumors tissue) (Reed and Purohit, 1997). The rate-limiting enzyme in the synthesis of estrogen in these tissues is cytochrome p450

aromatase (Brodie et al., 2001; Reed and Purohit, 1997). It is possible that alcohol increases systemic estrogen levels by affecting aromatase expression in these tissues. We show that alcohol increased the expression of aromatase in the tumors of alcohol-consuming mice, and this effect was more noticeable in NOVX mice than in OVX female mice.

Overall, we show that the promotion of tumor development by alcohol in MMTV-neu mice is associated with increased systemic estrogen levels and increased expression of aromatase and ERα in the tumors of NOVX mice. However, even though alcohol increased the expression of ERα in the OVX mice, alcohol did not increase in tumor incidence and tumor growth in these OVX mice. It is feasible that alcohol only affects HER2 tumor development in the presence of normal circulating estrogen levels, which our OVX mice lacked. On the other hand, systemic estrogen levels were elevated in NOVX mice, and this was associated with a higher tumor incidence and tumor growth rate. Therefore, it is plausible that alcohol consumption requires a certain threshold of systemic estrogen levels to promote mammary tumor development. This is supported by data showing that ovariectomy, which reduced systemic estrogen levels, inhibited the tumor-promoting effects of alcohol on MMTV-neu mice.

In conclusion, alcohol may promote HER2 mammary tumor development via the estrogen pathway. Interestingly, alcohol mediates the expression of ERα in HER2 tumors, which are reported to be ERα negative. Thus, drug therapies targeting the estrogen pathway may be able to block the effects of alcohol on an otherwise ERα-negative breast cancer. Given that many women drink some form of alcoholic beverage frequently, it is important to understand how alcohol consumption promotes breast cancer development in pre- and postmenopausal women. Determining the mechanism by which alcohol affects breast cancer may provide a therapeutic strategy to prevent alcohol-related breast cancers in women. In terms of the impact on breast cancer prevention, our findings may help define better recommendations on alcohol consumption for women to decrease their risk of developing this disease.

ACKNOWLEDGMENTS

The authors would like to acknowledge the ACS award ACS RSG CNE-113703 (NPN) for their financial support. We also wish to thank Jenny Lee, Tina Chiang, Emily Schrader, and Karen Poh for their assistance in animal care.

REFERENCES

American Cancer Society. 2010. Cancer facts & figures. Available from: http://www.cancer.org/acs/groups/content/@nho/documents/document/acspc-024113.pdf. Accessed March 1, 2011.

Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronada E, Shepard HM, Osborne CK (1992) Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat 24:85–95.

Brisson J, Verreaul R, Morrison AS, Tennina S, Meyer F (1989) Diet, mammographic features of breast tissue, and breast cancer risk. Am J Epidemiol 130:14–24.

Brodie AM, Lu Q, Long BJ, Fulton A, Chen T, Macpherson N, DeJong PC, Blankenstein MA, Nortier JW, Slee PH, van de Ven J, van Gorp JM, Elbers JR, Schipper ME, Blijham GH, Thijssen JH (2001) Aromatase and COX-2 expression in human breast cancers. J Steroid Biochem Mol Biol 79:41–47.

Byrne C, Schairer C, Wolfe J, Parekh N, Salane M, Brinton LA, Hoover R, Haile R (1995) Mammographic features and breast cancer risk: effects with time, age, and menopause status. J Natl Cancer Inst 87:1622–1629.

Cardiff RD (1998) Are the TDLU of the human the same as the LA of mice? J Mammary Gland Biol Neoplasia 3:3–5.

Chen WY, Colditz GA, Rosner B, Hankinson SE, Hunter DJ, Manson JE, Stampfer MJ, Willett WC, Speizer FE (2002) Use of postmenopausal hormones, alcohol, and risk for invasive breast cancer. Ann Intern Med 137:798–804.

Chung KW (1990) Effects of chronic ethanol intake on aromatization of androgens and concentration of estrogen and androgen receptors in rat liver. Toxicology 62:285–295.

Dorgan JF, Baer DJ, Albert PS, Judd JT, Brown ED, Corle DK, Campbell WS, Hartman TJ, Teipar AA, Clevidence BA, Giffen CA, Chandler DW, Stanczyk FZ, Taylor PR (2001) Serum hormones and the alcohol–breast cancer association in postmenopausal women. J Natl Cancer Inst 93:710–715.

Dumitrescu RG, Shields PG (2005) The etiology of alcohol-induced breast cancer. Alcohol 35:213–225.

Etique N, Chardard D, Chesnel A, Flament S, Grillier-Vuissoz I (2006) Analysis of the effects of different alcohols on MCF-7 human breast cancer cells. Ann NY Acad Sci 1030:78–85.

Etique N, Chardard D, Chesnel A, Merlin JL, Flament S, Grillier-Voissoz I (2004) Ethanol stimulates proliferation, ERalpha and aromatase expression in MCF-7 human breast cancer cells. Int J Mol Med 13:149–155.

Fan S, Meng Q, Gao B, Grossman J, Yadegari M, Goldberg ID, Rosen EM (2000) Alcohol stimulates estrogen receptor signaling in human breast cancer cell lines. Cancer Res 60:5635–5639.

Gapstur SM, Potter JD, Sellers TA, Folsom AR (1992) Increased risk of breast cancer with alcohol consumption in postmenopausal women. Am J Epidemiol 136:1221–1231.

Ginsburg ES (1999) Estrogen, alcohol and breast cancer risk. J Steroid Biochem Mol Biol 69:299–306.

Ginsburg ES, Mello NK, Mendelson JH, Barbieri RL, Teoh SK, Rothman M, Gao X, Sholar JW (1996) Effects of alcohol ingestion on estrogens in postmenopausal women. JAMA 276:1747–1751.

Gordan GG, Southren AL, Vittek J, Lieber CS (1979) The effect of alcohol ingestion on hepatic aromatase activity and plasma steroid hormones in the rat. Metabolism 28:20–24.

Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci USA 89:10578–10582.

Key TJ, Appleby PN, Reeves GK, Roddam A, Dorgan JF, Longcope C, Stanczyk FZ, Stephenson HE Jr, Falk RT, Miller R, Schatzkin A, Allen DS, Fentiman IS, Key TJ, Wang DY, Dowsett M, Thomas HV, Hankinson SE, Toniolo P, Akhmedkhanov A, Koenig K, Shore RE, Zeleniuch-Jacquotte A, Berrino F, Muti P, Micheli A, Krogh V, Sieri S, Pala V, Venturelli E, Secreto G, Barrett-Connor E, Laughlin GA, Kabuto M, Akiba S, Stevens RG, Neriishi K, Land CE, Cauley JA, Kuller LH, Cummings SR, Helzlsouer KJ, Alberg AJ, Bush TL, Comstock GW, Gordon GB, Miller SR, Longcope C, Endogenous Hormones Breast Cancer Collaborative Group (2003) Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. J Natl Cancer Inst 95:1218–1226.

Kwan ML, Kushi LH, Weltzien E, Tam EK, Castillo A, Sweeney C, Caan BJ (2010) Alcohol consumption and breast cancer recurrence and survival among women with early-stage breast cancer: the life after cancer epidemiology study. J Clin Oncol 28:4410–4416.

Lapidus R, Nass S, Butash K, Parl F, Weitzman S, Graff J, Herman J, Davidson N (1998) Mapping of ER gene CpG island methylation-specific polymerase chain reaction. Cancer Res 58:2515–2519.

Li Y, Yuan Y, Meeran SM, Tollefsbol TO (2010) Synergistic epigenetic reactivation of estrogen receptor-α (ERα) by combined green tea polyphenol and histone deacetylase inhibitor in ERα-negative breast cancer cells. Mol Cancer 9:274.

Li Y, Zhang Y, Shen Q, Kim HT, Xu X, Hilsenbeck SG, Bissonnette RP, Lamph WW, Brown PH (2007) The Rexinoid LG100268 prevents the development of preinvasive and invasive estrogen receptor negative tumors in MMTV-erbB2 mice. Clin Cancer Res 13:6224–6231.

McKinlay SM (1996) The normal menopause transition: an overview. Maturitas 23:137–145.

Menard S, Aiello P, Tagliabue E, Rumio C, Lollini PL, Colnaghi MI, Balsar A (2000) Tamoxifen chemoprevention of a hormone-independent tumor in the proto-neu transgenic model. Cancer Res 60:273–275.

Mill CP, Chester JA, Riese DJ (2009) EGFR may couple moderate alcohol consumption to increased breast cancer risk. Breast Cancer (London) 2009:31–38.

Nahta R, Shabaya S, Ozbay T, Rowe DL (2009) Personalizing HER2-targeted therapy in metastatic breast cancer beyond HER2 status: what we have learned from clinical specimens. Curr Pharmacogenomics Person Med 7:263–274.

Ottaviano Y, Issa J, Parl F, Smith H, Baylin S, Davidson N (1994) Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res 54:2552–2555.

Pasqualin JR (2002) Breast Cancer: Prognosis, Treatment, and Prevention. Marcel Dekker, New York, NY.

Peng J, Jordan VC (2008) Estrogen receptor pathways and breast cancer, in *Principles of Molecular Oncology* (Bronchud MH, Foote M, Giaccone G, Olopade O, Workman P 3rd eds), pp 189–203. Humana Press, Totowa, NJ.

Peyrat JP, Louchez MM, Lefebvre J, Bonneterre J, Vennin P, Demaille A, Helquet B, Fournier C (1993) Plasma insulin-like growth factor-1 (IGF-1) concentrations in human breast cancer. Eur J Cancer 29A:492–497.

Purohit V (2000) Can alcohol promote aromatization of androgens to estrogens? A review. Alcohol 22:123–127.

Reed MJ, Purohit A (1997) Breast cancer and the role of cytokines in regulating estrogen synthesis: an emerging hypothesis. Endocr Rev 18:701–715.

Roodi N, Bailey L, Kao W, Verrier C, Yee C, Dupont W, Parl F (1995) Estrogen receptor gene analysis in estrogen receptor-positive and receptor-negative primary breast cancer. J Natl Cancer Inst 87:446–451.

Schlüter C, Duchrow M, Wohlenberg C, Becker MH, Key G, Flad HD, Gerdes J (1993) The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. J Cell Biol 123:513–522.

Singletary KW, Gapstur SM (2001) Alcohol and breast cancer: review of epidemiologic and experimental evidence and potential mechanisms. JAMA 286:2143–2151.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neuoncogene. Science 235:177–182.

Smith-Warner SA, Spiegelman D, Yaun SS, van den Brandt PA, Folsom AR, Goldbohm RA, Graham S, Holmberg L, Howe GR, Marshall JR, Miller AB, Potter JD, Speizer FE, Willett WC, Wolk A, Hunter DJ (1998) Alcohol and breast cancer in women: a pooled analysis of cohort studies. JAMA 279:535–540.

State of Iowa Alcoholic Beverages Division. 2011. Blood alcohol content chart. Available from: http://www.iowaabd.com/alcohol/publications/bac_chart. Accessed March 1, 2011.

Stolier AJ, Wang J (2008) Terminal duct lobular units are scarce in the nipple: implications for prophylactic nipple-sparing mastectomy: terminal duct lobular units in the nipple. Ann Surg Oncol 15:438–442.

Tan H, Zhong Y, Pan Z (2009) Autocrine regulation of cell proliferation by estrogen receptor-alpha in estrogen receptor-alpha-positive breast cancer cell lines. BMC Cancer 9:31.

Werner H, Bruchim I (2009) The insulin-like growth factor-I receptor as an oncogene. Arch Physiol Biochem 115:58–71.

Wu K, Zhang Y, Xu XC, Hill J, Celestino J, Kim HT, Mohsin SK, Hilsenbeck SG, Lamph WW, Bissonette R, Brown PH (2002) The retinoid X receptor-selective retinoid, LGD1069, prevents the development of estrogen receptor-negative mammary tumors in transgenic mice. Cancer Res 62:6376–6380.

Yang X, Ferguson A, Nass S, Phillips D, Butash K, Wang S, Herman J, Davidson N (2000) Transcriptional activation of estrogen receptor alpha in human breast cancer cells by histone deacetylase inhibition. Cancer Res 60:6890–6894.

Yang X, Phillips D, Ferguson A, Nelson W, Herman J, Davidson N (2001) Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. Cancer Res 61:7025–7029.

Zhang SM, Lee I, Manson J, Cook NR, Willett WC, Buring JE (2007) Alcohol consumption and breast cancer risk in the women's health study. Am J Epidemiol 165:667–676.