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Original Article

Electronic cigarettes promotes the lung colonization of human breast cancer in NOD-SCID-Gamma mice

Do Huynh[1], Jing Huang[1], Le Thi Thu Le[1], Dongfang Liu[2], Chen Liu[1,2], Kien Pham[2], He Wang[1]

[1]Department of Pathology and Laboratory Medicine, Robert Wood Johnson Medical School, Rutgers University, New Brunswick, NJ; [2]Department of Pathology, Immunology & Laboratory Medicine, New Jersey Medical School, Rutgers University, New Brunswick, NJ

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Abstract: Electronic cigarettes (E-cigs) smoking or vaping is an emerging problem to public health due to its popularity. While its multi-faceted detrimental effects on human health are being reported, no current study addresses the effect of E-cigs on tumor metastasis, the main cause of tumor mortality. Using a well-established human breast cancer cell line MDA MB-231, we first showed that E-cig vapor extract (nicotine 24 mg/ml, propylene glycol 50%, vegetable glycerin 50%, no flavorings) significantly enhanced tumor cell migration ($P<0.0001$), but showed no significant effect on tumor cell proliferation ($P>0.05$). To evaluate the metastasis-promoting effect of E-cigs *in vivo*, we used NOD-SCID-Gamma mice and introduced tumor cells to the mice by tail vein injection. Among these mice, 4-week E-cigs exposure (nicotine 24 mg/ml, propylene glycol 50%, vegetable glycerin 50%, no flavorings, 2 h/day, 5 days/week) almost doubled the tumor load in the exposed lungs compared to controls ($P=0.0036$). While E-cig exposure did not alter the proliferative index of tumor cells colonized in the lungs ($P=0.7953$), tumor cell apoptosis was significantly reduced ($P<0.001$). Taken together, our data for the first time, demonstrated the lung colonization-promoting effects of E-cigs on human breast cancer cells. These findings show the risks of E-cigs on the lung metastasis of various cancers, and warrant more studies on the underlying mechanisms.

Keywords: Electronic cigarettes, lung colonization, human breast cancer, NOD-SCID-Gamma, metastasis

Introduction

E-cigarettes (E-cigs) were introduced to the United States market in 2007, initially as a safe alternative to traditional cigarette smoking (CS). E-cig liquid contains vehicle solvents such as vegetable glycerin (VG) and propylene glycol (PG), nicotine, and sometimes flavors [1]. Since its introduction, E-cigs have experienced widespread acceptance among smokers and non-smokers of all ages, but are particularly popular among youth. Emerging evidence suggests that E-cig use induces systematic inflammation and the activation of immune cells [1]. Previous studies showed that E-cig exposure induces DNA adduct formation, including mutagenic O^6-methyldeoxyguanosines and γ-hydroxy-propano-deoxyguanosine in mice lungs, heart and bladder, as well as enhances spontaneous lung carcinogenesis [2-4]. However, the impacts of E-cigs on tumor metastasis remain largely unknown.

Breast cancer is the most commonly diagnosed cancer among women in the United States [5]. It is well established that recurrence and metastasis are primary causes of breast cancer morbidity and mortality [6]. CS is highly associated with development and progression of breast cancer [7-9]. However, the mechanisms for CS-induced breast cancer lung metastasis remains elusive [10]. Furthermore, to the best of our knowledge, no literature addresses the effects of E-cigs on lungs metastasis of breast cancer. Therefore, in this study we investigated the effects of E-cig smoking on lung colonization of circulating breast cancer cells, a critical step in lung metastasis. Our results provide, for the first time, evidence E-cigs promote lung metastasis of human breast cancer cells. This is a significant contribution to our understanding of potential risk of E-cigs to human health and warrants further studies.

Materials and methods

Cell culture and reagents

Human breast cancer cell line MDA-MB 231 LM2 were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FBS, and 1% penicillin/streptomycin, maintained at 37°C in a humidified atmosphere of 5% CO_2, and tested negative for mycoplasma contamination. Media and supplements were purchased from Life Technologies (Carlsbad, CA, USA).

E-cig vapor condensate (EVC) preparation

For *in vitro* studies, E-cig commercial mix (PG and VG ratio is 50:50) was puffed and the extract was dissolved in cell culture medium and frozen at -80°C for further assays. The final concentration of nicotine in EVC was 80 ng/ml. For *in-vivo* studies, nicotine 100 mg/mL mixed with 50% propylene glycol (PG), and 50% vegetable glycerin (VG) to a final concentration of 24 mg/mL E-liquid.

Animal studies

Four-week-old female NOD-SCID-Gamma (NSG) mice were purchased from Jackson laboratory (ME, USA) and housed under specific pathogen-free conditions in a temperature-controlled and humidity-controlled room at Cancer Institute of New Jersey. Mice were exposed five days/week and two-hours/day to E-cigs (24 mg/mL Nicotine) by Inexpose system (SCIREQ, Quebec, Canada). For the air control group, mice were kept at normal condition. 5×10^5 cancer cells labelled with Luciferase and green fluorescent protein (GFP) reporter genes were intravenously (i.v.) injected into tail vein. Lung metastasis was monitored weekly by intraperitoneally (i.p.) injecting D-luciferin (Biovision, CA, USA) at dose 100 mg/kg and detected by IVIS imaging system (Perkinelmer, MA, USA). At the end point, mice were sacrificed; mice blood and lungs were collected for further assays.

Urine cotinine assay

Urine samples from mice exposed to or without E-cigs were used for cotinine measurement by murine/rat cotinine assay kit (CalBiotech, CA, USA). In brief, urine was mixed with 100 µL enzyme conjugate to each well and incubated 1 h at room temperature/dark room. Wells were washed with distilled water, to which was added 100 µL substrate, followed by 30 min incubation in the dark. 100 µL Stop solution was added before reading at 450 nm.

Immunohistochemical staining

Lungs from the mice were obtained, perfused and fixed by Formalin 10% before paraffin-embedding. After deparaffinization and heat-induced epitope retrieval, the sections at 5 µm were blocked with normal goat serum and incubated with anti-GFP, anti-Ki67, and anti-cleaved caspase-3 at 4°C overnight. All antibodies were purchased from Abcam (Cambridge, UK). Sections were then rinsed with phosphate-buffered saline (PBS), incubated with secondary antibody for 1 h. dehydrated and sealed, then images were captured using a Olympus BX43 microscope (Olympus, Tokyo, Japan).

Cell proliferation assay

Human breast cancer cells MDA-MB 231 LM2 were seeded onto 96-well plates at 3.0×10^3 cells/well. Cells were treated with various concentrations of E-cigs. After 24 h treatment, 10 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich, MO, USA) was added into the wells and incubated for 4 h before reading at 570 nm by microreader.

Transwell migration assay

After treatment with E-cigs, 0.5% (0.4 ng/mL) 24 h, 200 µL of cells in 0.5% FBS medium were seeded into the upper chamber (8 µm) of a Millicell hanging cell culture insert (Millipore-Sigma, MA, USA), at a density of 2.5×10^4 cells/well. The lower chamber was added with 800 µL culture medium supplemented with 10% FBS. After 24 h, the cells on the upper surface of the membrane were removed with a cotton swab and the cells in lower chamber were fixed with paraformaldehyde 4% (PFA) and stained with crystal violet for 30 min. The number of migrated cells was counted in five random areas under a microscope.

Statistical analysis

All data are presented as the mean ± SD (standard deviation). The significances of differences between groups were analyzed using Stu-

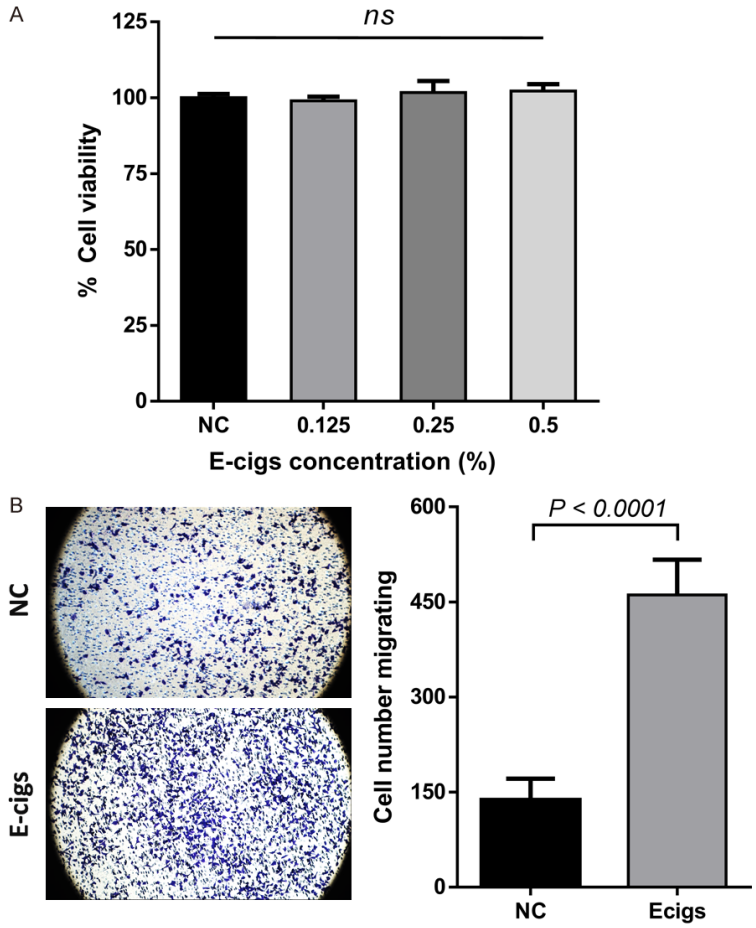

Figure 1. E-cigarettes enhance cell mobility in MDA-MB 231 LM2. A. Cell proliferation assay of MDA-MB 231 LM2 under E-cigarette extract treatment. B. Migration assay of MDA-MB 231 LM2 under E-cigarette extract treatment (left); Quantification of cell numbers migrating (P<0.001, right).

dent's *t*-test, one-way, or two-way ANOVA. Values of $P<0.05$ were considered significant. All the experiments were repeated at least three times.

Results

E-cigs enhance the mobility of breast cancer cells in vitro

We first performed a cell viability assay to examine the optimal E-cig dose on human MDA-MB 231 LM2 cells. As shown in **Figure 1A**, treatment with E-cig at concentrations ranging from 0-0.5% (0-0.4 ng/ml) did not significantly alter the cell numbers of MDA MB231. Next, we tested the effects of E-cigs on cell mobility. As shown in **Figure 1B**, E-cig treatment (0.4 ng/ml) increased the number of migrated MB 231 LM2 cells by 3 fold ($P<0.0001$).

E-cig inhalation increases urine cotinine level in mice

Cotinine is the secondary metabolite of nicotine. Its existence in our body represents the environmental smoking and serves as a marker to distinguish between smokers and non-smokers [11]. In this study, we exposed the mice to E-cigs and urine samples were collected at the end of study. Our result showed that level of urine cotinine in E-cigs treatment was significantly higher than that of air control mice (approximately 30 folds, $P=0.0024$) (Supplementary Figure 1).

Gross and IVIS examination of lung colonization of breast cancer cells in vivo

To evaluate the possibility that E-cig exposure accelerates breast cancer metastasis, we used Luciferase-GFP labelled human breast cancer cells were intravenous injected into NSG mice, followed by exposing with E-cigs by InExpose system and the metastasis were examined weekly by IVIS system. Results showed that the lung localization of tumor cells in mice exposed to E-cigs was clearly higher than that of the air controls (**Figure 2A**). Furthermore, gross examination revealed no prominent nodules on lung surfaces in either E-cig or air-exposed groups (**Figure 2B**).

Immunohistological analysis of lung colonization of breast cancer cells

To evaluate the possibility that E-cigs promote breast tumor cell colonization to lungs at the microscopic level, whole mice lungs were sectioned and stained with GFP, which labels tumor cells. Double immunohistochemical stains showed that level of positive GFP cells or tumor areas were very increased as compared to air controls (**Figure 3A**, top). However, human breast cancer proliferation after E-cigs

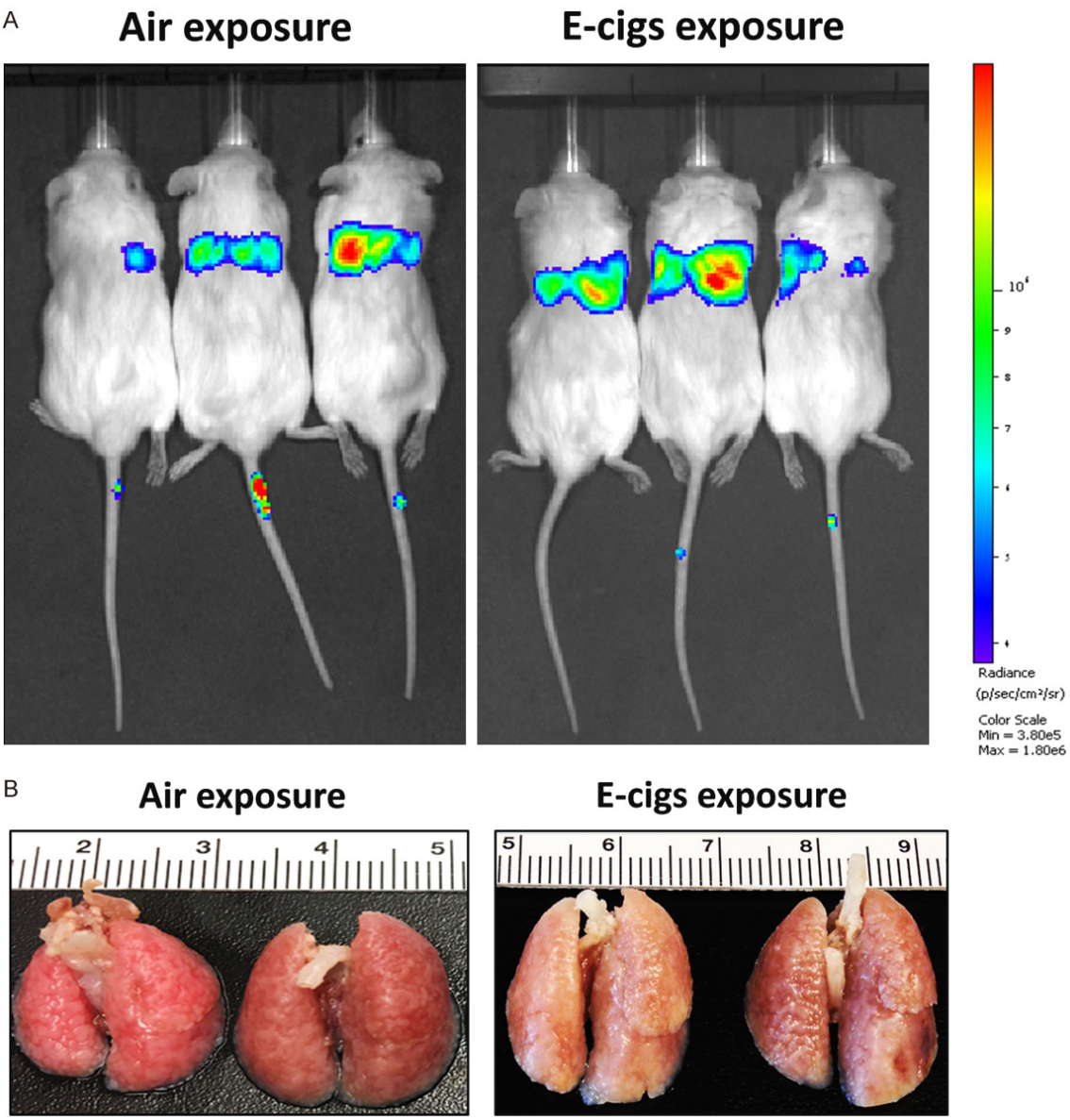

Figure 2. E-cigarettes accelerate lung colonization of breast cancer cells. A. Tumor cell localization detected by IVIS system. B. Lungs after 4 weeks' E-cigarette exposure.

treatment had no significant changes, illustrated by Ki67 staining (**Figure 3A**, middle). In addition, tumor apoptosis, demonstrated by cleaved caspase-3 stain, in lung colonized breast tumors of air-exposed mice was more notable than that of E-cig exposed mice (**Figure 3A**, bottom). Morphometric analysis indicated that tumor area in the E-cig treatment group was 2 fold greater than that of the air control group (*P=0.0036*) while Ki67 staining cells between the two groups were not different (**Figure 3B**). In addition, apoptotic GFP-positive cells in E-cig exposed lung sections were 5-fold lower as compared to those of the air exposed group (**Figure 3B**).

Discussion

Using an NSG mouse model, our data revealed that 4-week E-cig exposure significantly promoted the lung colonization of human breast cancer MDA-MB 231 LM2 cells. Double immunohistochemical staining illustrated a decreased apoptotic rate of metastasizing breast cancer cells under E-cig exposure. Parallel with the *in vivo* data, *in vitro* result showed that E-

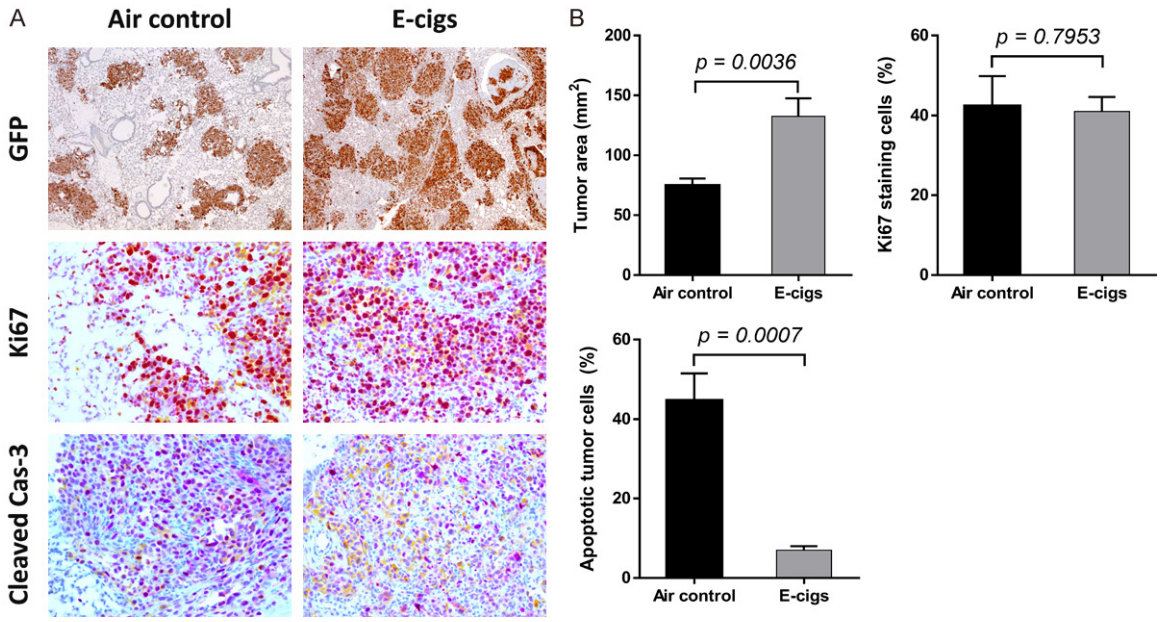

Figure 3. Immunohistochemical staining of lungs with and without E-cigarette treatment. A. Sections stained with GFP, Ki67, and cleaved Caspase-3 with and without E-cigarette treatment. B. Quantification of GFP, Ki67, and cleaved Caspase-3 signals with and without E-cigarette treatment (P=0.04, P=0.7953 and P<0.001 respectively).

cig extract treatment enhanced breast cancer cell migration ability while there was a negligible effect on tumor cell proliferation. Our results, for the first time, illustrates that E-cig smoking promotes lung colonization of circulating breast cancer cells.

Breast cancer is one of the most common causes of women's cancer death [5]. Current therapies are highly effective to treat early and non-metastatic stage breast cancer; but much less effective to inhibit late cancer progression or metastasis [6]. Evidence highlights a strong connection between traditional CS and cancer development and metastasis [7-9, 12, 13]. With the primary goal of minimizing the harm of traditional CS, E-cigs are expected to be a safe alternative [4]. However, the health-detrimental risks related to E-cigs uses are being quickly revealed [14]. Tang *et al.* indicate that E-cig exposure for 4 months accelerates DNA adduct formation in lungs and bladder of mice, a well-established risk factor for genome mutations and carcinogenesis [3]. Furthermore, the Tang group also illustrated that long-term exposure to E-cigs results in spontaneous lung adenocarcinomas (9 of 40 mice, 22.5%) and bladder urothelial hyperplasia (23 of 40 mice, 57.5%) in FVB/N mice [4]. However, the effects of E-cigs on BC metastasis and its underlying mechanisms remained elusive.

Di Cello *et al.* demonstrate that traditional CS promotes the transformation of human non-metastatic breast cancer cells and non-malignant mammary epithelial cells to highly metastatic and tumorigenic cells [12]. Their study shows that long-term exposure to either cigarette smoke extract (CSE) or cigarette smoke condensate (CSC) induces anchorage-independent growth and migration/invasion of mammary epithelial cell lines. Furthermore, CS extract-treated MCF-7 cells showed higher lung metastasis capability after mammary fat pad injection whereas no metastasis was identified in mice injected with untreated MCF-7 cells. These data suggest that E-cig use is able to trigger the mutations or tumorigenicity in benign cells, and traditional CS prompts the metastasis of breast cancer cells.

Molecular mechanisms underlying the higher migration rate of E-cig treated-breast cancer cells are being actively investigated by our team. Interactions of multiple pathways might be responsible for this phenomenon. First, E-cig treatment may act similarly to traditional CS and upregulate surface markers of breast cancer or induce epithelial to mesenchymal transition (EMT), which is necessary for lung colonization [15-19]. Second, E-cigs inhalation could induce oncogenic cytokine or microRNA release from both pulmonary cells and breast

cancer cells, consequently promoting lung colonization of breast cancer cells, similar to traditional CS [10]. A third possibility is that E-cig exposure might enhance the survival of breast cancer cells during invading and "nesting" processes. Evidences from the literature indicates that during metastasis, cancer cells are prone to apoptosis [20, 21]. This may come from the misplacing of tumor cells during metastasis, or by the loss of cell-cell and cell-ECM contacts. In addition, the metastatic cells may face attack by cytotoxic lymphocytes such as natural killer (NK) cells, which are resident at secondary sites such as lungs or liver. Furthermore, lack of nutrients (growth factors, glucose) and increase in intracellular reactive oxygen species (ROS) level might also induce death of cancer cells. Interestingly, double immunohistochemical analysis in our study shows that the apoptosis rates of colonized tumor cells are significantly decreased in the E-cig treatment group; suggesting the anti-apoptotic role of E-cigs in this setting.

Other than directly acting on tumor cells itself, E-cig use might also alter the function and/or number of immune cells in the tumor microenvironment. E-cig vapor condensate (EVC) significantly increases production of interleukin-6, tumor necrosis factor α, and matrix metallopeptidase-9 from cultured alveolar macrophages [22]; it suppresses cellular antioxidant defenses and triggers DNA damages in cultured respiratory epithelial cells [23]. In addition, chronic vaping is believed to induce protease release from resident immune cells of the pulmonary system [24]. Thus, it is possible that E-cigs inhaled into the body help to activate immune cells and boost the interaction between breast cancer cells and immune cells, leading to more or easier lung colonization.

As a first study to address the effect of E-cigs on breast cancer lung metastasis, there are limitations to current study. First, tail vein injection approach only addresses the later stage of cancer metastasis: colonization and tumor growth at a secondary site. For this reason, our results in this report focus only on lung colonization. A follow-up study with both mammary fat pad injection and tail vein injection are well underway in our lab. Preliminary results suggest E-cigs promote all stages of lung metastasis of breast cancer. Second, this study emphasizes the *in vivo* aspect of breast cancer metastasis, and highlights the tumor size, apoptosis, and proliferation. More detailed analysis with different exposure regimens (different nicotine concentrations, additions of E-cig flavorings) and more *in vitro* mechanism studies are absolutely necessary for future studies. Third, a human breast cancer cell line is used in our study. While this cell line is more relevant to human disease, adoption of severe immune deficient mouse model excludes the possibility of studying the role of immune cells in our system.

In summary, our results provide the first evidence that the E-cigs promote human breast cancer cell mobility *in vitro* and lung colonization *in vivo*. This is a significant contribution to our understanding of the risk of E-cigs to human health and warrants further studies on E-cigarette induced breast cancer metastasis.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. He Wang, Department of Pathology and Laboratory Medicine, Robert Wood Johnson Medical School, Rutgers University, MEB212, 1 Robert Wood Johnson Place, New Brunswick 08901, NJ. Tel: 732-507-8113; Fax: 732-235-8124; E-mail: hw423@rwjms.rutgers.edu; Dr. Kien Pham, Department of Pathology, Immunology & Laboratory Medicine, New Jersey Medical School, Rutgers University Newark 07103, NJ. Tel: 3522263652; Fax: 3522263780; E-mail: ktp52@njms.rutgers.edu

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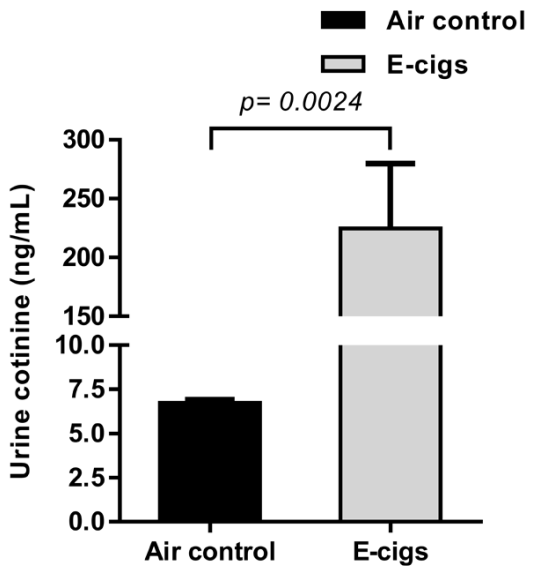

Supplementary Figure 1. E-cigarettes increase urine cotinine in mice (P=0.0024).