Effects of cigarette smoke extract derived from heated tobacco products on the proliferation of lung cancer stem cells

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ABSTRACT
Epidemiological studies have suggested that cigarette smoking can increase a person’s risk of developing several types of cancer, including lung cancer. Lung cancer originates from cancer stem cells (CSCs), which constitute a minor cell population in tumors, and contribute to drug resistance and recurrence. Heated tobacco products (HTPs) produce aerosols that contain nicotine and toxic chemicals. Current evidence, however, is insufficient to accurately determine if HTPs are less harmful than burned cigarettes. This study has investigated the effects of cigarette smoke extract (CSE) from HTPs on lung CSCs in lung cancer cell lines. We found that CSEs induced the proliferation of lung CSCs and increased the expression levels of stem cell markers. In addition, CSE induced epithelial-mesenchymal transition (EMT) expression and cytokine production. These results suggest that HTPs can induce lung CSCs in vitro.

1. Introduction
Epidemiological studies have suggested that cigarette smoking is a risk factor for carcinogenesis and the progression of several types of cancer, including lung cancer. Cigarette smoke contains over 5000 chemicals, including 79 classified as oncogenic [1,2]. The effects of cigarette smoking on the health of active and passive smokers is both socially and medically important.

Evidence has increasingly suggested that lung cancer originates from cancer stem cells (CSCs), which constitute a minor cell population in tumors and contribute to cellular heterogeneity [3]. Since CSCs have the potential for high levels of drug resistance and tumorigenicity, they are considered to be associated with cancer development and relapse. Aldehyde dehydrogenase (ALDH), a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, has recently been used as a functional marker to identify CSCs in clinical samples and cancer cell lines [4]. We have previously reported that nicotine induces the proliferation of breast CSCs in MCF-7 [5] and that tobacco-specific nitrosamine 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) induces the proliferation of lung CSCs in A549 [6].

Heated tobacco products (HTPs) are relatively new, and produce aerosols containing nicotine and other chemicals via vapor. There are several HTPs that are commercially available, including Glo by British American Tobacco Plc., IQOS by Phillip Morris International Inc., and Ploom S by the Japan Tobacco Inc. HTPs reportedly have reduced levels of harmful chemicals, such as nicotine, tar, and carbonyl compounds, when compared to conventional cigarettes [7,8]. However, HTPs are also known to have increased levels of some chemicals, such as glycerol, propylene glycol, and glycidol, when compared with burned cigarettes [9,10]. As HTPs are now being sold globally, there is increasing attention regarding their associated health impacts. According to the World Health Organization (WHO), there is currently insufficient evidence to conclude that HTPs are less harmful than burned cigarettes. Thus, the relationship between HTPs and cancer development is not yet fully understood.

We have investigated the effects of cigarette smoke extract (CSE) from HTPs on lung CSCs in vitro. We found that the CSE from HTPs induces CSC proliferation, and that CSE induced both stem cell and epithelial-mesenchymal transition (EMT) markers.

Abbreviations: CSCs, cancer stem cells; HTPs, heated tobacco products; CSE, cigarette smoke extract; EMT, epithelial-mesenchymal transition; ALDH, aldehyde dehydrogenase; NNK, 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone; WHO, World Health Organization.

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2. Materials and methods

2.1. Cell culture

A549 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), while the HCC827 and H1975 cells were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco BRL, Carlsbad, CA, USA).

2.2. Preparation of the CSE from the HTPs

CSE from three types of HTPs was prepared in accordance with previously reported methods [11]. CSE from Glo, IQOS, and Ploom S are coded as HTPa, HTPb, and HTPc, respectively.

2.3. ALDH assay

The ALDEFLUOR kit (Stem Cell Technologies, Vancouver, BC, Canada) was used to detect CSC populations with high ALDH enzyme activity as was previously reported [12]. The cells were plated at a density of $1 \times 10^5$ cells in 60 mm culture dishes. After serum deprivation...
overnight, the cells were treated with each concentration of the CSE for three days. Cells were then suspended at a concentration of $1 \times 10^6$ cells/mL in an ALDH assay buffer containing the ALDH substrate BAAA (1 $\mu$M) and incubated for 30 min at 37 $^\circ$C. As a negative control, cells were treated with diethylaminobenzaldehyde (DEAB, 15 $\mu$M), a specific ALDH inhibitor. A FACS Aria II cell sorter (BD Biosciences, San Jose, CA, USA) was used to measure and sort the ALDH-positive and -negative cells.

2.4. Sphere-forming assay

The sphere-forming assay was performed as previously described [12]. Briefly, cells were plated on ultra-low attachment 24-well plates (Corning, Acton, MA, USA) at a density of 5000 cells/mL in serum-free DMEM supplemented with $\text{N}_{2}$ (Gibco), 20 ng/mL basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA), and each concentration of the CSE. After 5 days, the number of spheres was analyzed microscopically. Images of spheres were taken at 100x magnification.
Fig. 3. Effects of the CSEs derived from the HTPs on the sphere formation. (A) Representative images of sphere formation. Images of spheres were taken at 100x magnification. Bar = 20 µm. (B–D) Effects of HTPa (B), HTPb (C), and HTPc (D) on the sphere formation in lung cancer cell lines. Data are presented as the means ± SD (n = 3). *p < 0.05.
2.5. qRT-PCR

After the cells were treated with CSE for 24 h, total RNA was isolated using the TRIzol reagent (Thermo, Waltham, MA, USA), according to the manufacturer’s instructions. qPCR assays were conducted using a QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Valencia, CA, USA) and a ViiA7 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative changes in transcript levels for each sample were determined by normalization to the GAPDH mRNA levels [13,14]. Primer sequences are listed in Supplementary Table S1.

2.6. Statistical analysis

Results are shown as the mean ± s.d and statistical analyses were performed using Excel 2010. P values were calculated using a two-sided unpaired Student’s t-test. Differences were considered statistically significant at P < 0.05 Fig. 1.

3. Results

3.1. CSE from HTPs induces the proliferation of ALDH-positive cells

Firstly, we examined the cytotoxicity of CSE prepared from three types of HTP (HTPa, HTPb, and HTPc) in lung cancer cell lines (A549, HCC827, and H1975). HTPa at 0.3% and 1% slightly inhibited the proliferation of A549 cells. HTPb at 0.3% or more inhibited the proliferation of A549 cells. HTPc at 0.3% slightly inhibited the proliferation of A549 cells. In HCC827 and H1975, HTPs did not have significant effects on the proliferation. To examine the effects of the CSE from HTPs on the CSCs, we performed an ALDH assay using lung cancer cell lines (A549, HCC827, and H1975). HTPa induced the proliferation of ALDH-positive cells in a dose-dependent manner in A549 cells, HCC827 and H1975 cells (Fig. 2A). HTPb was also found to induce the proliferation of ALDH-positive cells in all three cell types (Fig. 2B). In addition, HTPc induced the proliferation of ALDH-positive cells in A549 and HCC827 cell lines (Fig. 2C). These results suggest that CSE...
induces the proliferation of lung ALDH-positive cells.

### 3.2. CSE from HTP induces sphere formation

To confirm the effects of the CSE from the HTP on the CSCs, we assessed their sphere-forming ability using another CSC assay (Fig. 3A). Like the ALDH assay, HTPa induced sphere formation in the three cell lines in a dose-dependent manner (Fig. 3B). HTPb induced sphere formation in the A549 cells (Fig. 3C), while HTPc induced sphere formation in HCC827 and H1975 cells (Fig. 3D). These results confirm the effects of the CSE from the HTPs on the proliferation of lung CSCs.

### 3.3. CSE from HTP induces the expression of stem cell markers

To investigate the molecular mechanisms by which the CSE from the HTPs induced the proliferation of CSCs, we analyzed the stemness markers Oct3/4, Nanog, and Sox2. HTPa (3% in three cell lines) and HTPb (0.1% in A549 and HCC827, and 3% in H1975) significantly increased the ALDH-positive cells. Because HTPc did not significantly increase the ALDH-positive cells, we selected 3% HTPc. Similar to the ALDH assay, HTPa upregulated the expression of the three stem cell markers in the three cell lines (Fig. 4A). HTPb also upregulated the expression of stem cell markers, except for Sox2 in the A549 and HCC827 cells (Fig. 4B). In contrast, HTPc upregulated the expression of stem cell markers in HCC827 cells (Fig. 4C). These results suggest that the CSE from the HTPs increased the proliferation of ALDH-positive cells via different mechanisms.

### 3.4. CSE from the HTPs induces the expression of EMT markers

Accumulating evidence suggests that CSCs exhibit EMT potential [15]; consequently, we analyzed whether HTPs could induce the expression of EMT markers. Like the ALDH assay and stem cell markers, HTPa upregulated the expression of Twist and Snail and decreased the expression of E-cadherin in the three cell lines, suggesting HTPa induced EMT (Fig. 5A). HTPb induced EMT in the HCC827 and H1975 cells but...
not in the A549 cells (Fig. 5B). In contrast, HTPc did not induce EMT (Fig. 5C). These results suggest that the effects of the CSE from the HTPs on the EMT differ among the HTPs.

3.5. CSE from HTP induced the expression of inflammatory cytokine

To examine whether inflammation was involved in CSE-induced EMT, we analyzed the expression of inflammatory cytokines. HTPa induced the expression of both IL-6 and IL-8 in all three cell lines (Fig. 6A). HTPb induced the expression of both IL-6 and IL-8 in H1975 cells (Fig. 6B). HTPb induced only IL-6 expression in HCC827 cells (Fig. 6B). In the case of HTPc, IL-6 and IL-8 levels were not induced (Fig. 6C). These results suggest that cytokine production by HTPs is similar to stemness and EMT induced by HTPs.

4. Discussion

In the present study, we found that CSEs from HTPs induced the proliferation of lung CSCs and the expression of CSC markers. In addition, the CSEs from the HTPs induced the expression of EMT markers and inflammatory cytokines, suggesting that HTPs contain harmful chemicals in their aerosols that could affect the development of lung cancer. These data suggest that HTPs are associated with the development of lung cancer.

We found that the three types of CSE derived from the HTPs all induced cell proliferation. The effects were slightly different among the HTPs, as each was made at a different temperature (HTPa (240 °C) < HTPb (300–350 °C) < HTPc (200 °C)), suggesting that their chemical components differed. The components of the HTPs are also known to vary depending on the smoking conditions. It is necessary to consider the concentrations of the chemicals in the air and blood after smoking HTPs.

We have previously reported that tobacco-specific N-nitrosamine 4-(methylnitrosamino)–1-(3-pyridyl)–1-butanone (NNK) increases the number of ALDH-positive A549 cells via the activation of Wnt signaling [6]. Although NNK is known to be a carcinogen, it has been reported that amounts of NNK in HTPa and HTPb was decreased in comparison with conventional burned cigarettes [9,10]. HTPa and HTPb did not increase
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the expression of the Wnt target gene Dkk1 (Supplementary Fig. S1).
Consistent with the ALDH assay, we found that HTPa- and HTPb-induced EMT markers. Several components of cigarette smoke, such as nicotine, benzo[a]pyrene, formaldehyde, isoprene, acrylonitrile, and N’-nitrosonornicotine (NNN) are also reported to induce EMT markers [16–18]. These chemicals have also previously been detected in the mainstream aerosol of HTPa and HTPb [8–10]. Compared with HTPb, HTPa induced EMT markers significantly. Since the amount of NNN in HTPa mainstream aerosol has been more than that in HTPb mainstream aerosol [9,10], NNN might be a candidate chemical that induces EMT in HTPs. To date, only nicotine has been analyzed in the blood concentration chemicals of the HTPs smokers [19]. Future studies are required to analyze and identify other chemicals involved in the CSE-induced proliferation of lung CSC via EMT.

EMT has been shown to be regulated by inflammatory cytokines, such as IL-6 and IL-8 in several types of cancer, including lung cancer [20]. HTPs induced IL-6 and IL-8 expression in three cell lines, whereas HTPb induced them in the EGFR-mutated cell lines HCC827 and H1975, but not EGFR wild type cell line A549, suggesting that EGFR/MAPK signaling is necessary to induce cytokines by HTPb. Several chemicals, such as carbonyl compounds and metals, induce IL-8 expression via EGFR signaling [21,22]. Comparing the components of HTPs and HTPb may provide clues to help identify important chemicals.

In summary, the present study revealed that HTPs-induced lung CSCs and EMT. Previous data suggest that HTPs may progress to cancer development. Future studies are required to analyze the components of CSE in the HTPs and identify important chemicals.

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CRediT authorship contribution statement
Naoya Hirata: Formal analysis, Investigation, Writing – original draft, Visualization. Takahiro Horinouchi: Conceptualization, Resources, Writing – review & editing. Yasunari Kanda: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.06.001.

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