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Original Article

Detection of trichomonads in patients with lung cancer and transcription analysis on the response of human pulmonary epithelial cells to *Trichomonas tenax* invasion

Chun-Hsien Chen [a,b,1], Chin-Wei Kuo [c,d,1], Chih-Ming Tsai [b], Zih-Bin Hong [b], Ching-Han Lai [e], Tzu-Yi Chan [d], Wei-Chen Lin [a,b,*]

[a] *Department of Parasitology, College of Medicine, National Cheng Kung University, Tainan, Taiwan*
[b] *Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan*
[c] *Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan*
[d] *Division of Chest Medicine, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan*
[e] *Division of Critical Care Medicine, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan*

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KEYWORDS
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Abstract *Introduction:* Lung cancer is one of the most prevalent malignancies worldwide. Substantial research has illuminated the intricate interplay between microorganisms and human health, revealing their role in disease regulation. Trichomonads is a flagellated protozoan in the human cavity and have been previously identified as a pathogen associated with pneumonia, contributing to tissue chronic inflammation and carcinogenesis.
Methods: Nested polymerase chain reaction methods were employed to scrutinize the prevalence of trichomonads in the bronchovesicular fluid of patients diagnosed with lung cancer. Subsequently, the influence of *Trichomonas tenax* invasion on lung cancer cells was elucidated through proliferation assays, migration assays, and transcription analysis.
Results: Bronchoalveolar fluid samples from lung cancer patients yielded positive nested PCR results for eight out of twenty-seven samples. Seven of these samples were identified as *Trichomonas tenax*, while one was identified as *Tetratrichomonas* spp. Our findings revealed a significant upregulation of pathways associated with carcinogenesis, including cellular proliferation, migration, and drug resistance, in response to *T. tenax* invasion.

* Corresponding author. Department of Parasitology, National Cheng Kung University, No.1, University Road, Tainan City 701, Taiwan.
E-mail address: wcnikelin@mail.ncku.edu.tw (W.-C. Lin).
[1] The first two authors contributed equally.

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Conclusions: This study underscores the importance of recognizing the presence of trichomonads and the influence of *T. tenax* invasion on host responses to respiratory diseases. The identified pathways implicated in cancer development may pave the way for developing targeted treatment strategies for pulmonary diseases. These findings hold promise for informing and improving the precision of therapeutic interventions in the context of pulmonary ailments.

Introduction

Lung cancer ranked as the second most prevalent cancer globally and emerged as the foremost cause of cancer-related mortality.[1] In 2020, approximately 2.2 million new cases of lung cancer were estimated worldwide, constituting 11.4% of all newly diagnosed cancer cases.[1] In Taiwan, lung cancer represents the second most prevalent cancer, contributing to 39.1% of all newly diagnosed cancer cases.[2]

The correlation between microorganisms and human health involves several physiological processes, including nutrition metabolism and immune responses.[3] Recent findings have uncovered compelling associations between parasites and autoimmune diseases, metabolic disorders, and even cancer development.[3] *Clonorchis sinensis*, an important foodborne liver pathogen in East Asia, can induce cholangiocarcinoma, and *Schistosoma haematobium*, is a parasitic blood fluke, that may cause cancer of the urinary bladder.[4] Research suggests that parasites may participate in carcinogenesis through parasite-derived oxidative stress and chronic inflammation.[5] Trichomonads, a genus of parasitic flagellate protozoa, inhabit the digestive and reproductive systems of humans. *Tetratrichomonas* spp. and *Trichomonas tenax* have been identified in bronchoalveolar lavage fluid samples from patients with pulmonary diseases. *Trichomonas tenax* has been indicated a connection with periodontal disease.[6] The oral cavity serves as the entry point to the respiratory tract; its microbiome may play a role in the development and progression of pulmonary diseases.[7] Our research has conducted an investigation, providing evidence of the presence of oral protozoa, including *T. tenax*, in patients diagnosed with periodontitis in Taiwan.[8] Additionally, the invasion of Trichomonas invasion did not result in epithelium damage but the production of interleukin-6 (IL-6), a molecular link between proinflammatory cytokines and lung cancer, in pulmonary cells.[9] However, the prevalence and impact of Trichomonas invasion in pulmonary cancer remain inadequately understood. Thus, it is crucial to establish the presence of Trichomonas in patients with lung cancer and elucidate the host responses incited by Trichomonas invasion.

This study represents the first investigation wherein we unveiled the prevalence of *T. tenax* in bronchoalveolar lavage fluid samples obtained from patients with lung cancer in Taiwan, employing the DNA-based analysis. Subsequent investigations disclosed that co-incubation with trichomonas augmented the proliferation of pulmonary epithelial cancer cells. Through transcriptional analysis, we elucidated the responses of pulmonary cancer cells to *T. tenax* exposure. The results revealed a significant upregulation of pathways associated with cancer development, encompassing proliferation, migration, and drug resistance, upon Trichomonas invasion. In conclusion, our findings suggest that heightened awareness of protozoan presence and its influence on host responses in respiratory diseases potentially paving the way for the development of appropriate and targeted treatment strategies.

Methods

Study design and patients

To obtain bronchoalveolar lavage fluid (BALF) from patients with lung cancer, we prospectively collected BALF from participants who underwent bronchoscopic biopsy for lung cancer survey at the National Cheng Kung University Hospital (NCKUH) between July 2022 and April 2023. This study was conducted with the approval of the Institutional Review Board (A-ER-110-277) at the NCKUH. Patients with final diagnosis of lung cancer were included, the exclusion criteria were as follows: 1) age under 20 years old; 2) deterioration of inflammatory airway disease or pulmonary infection $<$1 month; 3) diagnosis of interstitial lung disease or diffuse pulmonary lesion; 4) receiving inhaled or systemic immunosuppressive therapy. Basic information about the participants, including demographics, clinical lung cancer diagnosis, and cancer stage was collected from medical records in the NCKUH database.

Bronchoalveolar lavage fluid collection and preparation

These BALF samples were obtained through fiberoptic bronchoscopy with efforts made to minimize oral contact. The procedure was performed following recommended practice guidelines,[10] and the samples were processed by researchers who were blinded to the clinical data. Each patient received normal saline aliquots, with the total volume of instillation ranging from 100 to 300 mL and the retrieval volume ranging from 30 to 50 mL, respectively. The collected BALF was filtered using a 70 μm strainer and then centrifuged at 1500$\times g$ for 5 min. After centrifugation, the supernatant was removed, and the pellets of samples were suspended with PBS for genomic DNA extraction.

DNA extraction and nested polymerase chain reaction

Specimen genomic DNA was extracted using a QIAamp DNA Mini Kit (Hilden, Germany) from 80 μl of BALF sediment. The 18S rRNA gene was amplified by nested PCR to elevate the sensitivity. The primers and reaction conditions were followed by previous study.[11] The PCR products were subjected to 1.5% agarose gel electrophoresis, and the target bands (approximately 390 bp) were isolated for subsequent sequencing using TRC2 primers. The sequence results were aligned to identify the species of trichomonads by BLAST tools in the Genebank.

Trichomonas tenax and cell culture

Trichomonas tenax, ATCC_30207, was obtained from the American Type Culture Collection. Trichomonas was cultured in yeast extract-iron-serum medium with 10% horse serum (Gibco™, Thermo Fisher Scientific) under anaerobic condition at 37 °C.

Mucoepidermoid Pulmonary cells NCI—H292 (ATCC_CRL-1848), obtained from the American Type Culture Collection, were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum and 100 units/mL penicillin-streptomycin (Thermo Fisher Scientific). The cells were maintained as monolayers at 37 °C and 5% CO_2 in a routine culture.

Cell proliferation assay

For cell proliferation assay, NCI—H292 was seeding at at a density of 4×10^3 cells per well in 96-well plate overnight. *T. tenax* was harvested from the routine culture medium and resuspended in RPMI. *T. tenax* at the density of multiplicity of infection (MOI) 2 was added into wells. The control group were also replaced with fresh RPMI. CCK8 reagents were added into each well after 24- or 48-h co-incubation.

Cell migration assay

The migration assay was performed using transwell cambers system with an 8-μm pore size polycarbonate membrane inserts (Corning, USA). NCI—H292 cells were cultured at a density of 4×10^4 cells per insert in a 24-well plate. The NCI—H292 cells were cultured with or without *T. tenax* in the upper chambers of the 24-well plate (Corning, USA) in a serum-free medium. The cells were incubated at 37 °C for 24 h. At the end of the experiment, the cells were fixed with methanol and stained with Giemsa, then cells on the upper surface of the membrane were removed with a cotton swab. The images were taken with an inverted microscope (CX41, Olympus) and analyzed using NIH ImageJ software.[12] Each experiment was performed in triplicate.

RNA extraction and real-time PCR

NCI—H292 cells were cultured at a density of 1.5×10^6 cells per well in a 6-well plate. The number of *T. tenax* is based on our previous study. Pulmonary cells incubated with or without *T. tenax* at 37 °C for 24 h were lysed by adding TRI Reagent®. The Direct-zol TM RNA Mini Prep Kit (Zymo Research) was used for RNA extraction. The total RNA was then stored at −80 °C. The concentration and purity (A 260/A 280 ratio) of the total RNA were quantified using a NanoDrop TM 1000 instrument (Thermo Fisher Scientific, USA). To quantify the gene expression of treated cells, reverse transcription (RT)-PCR was used by the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Primers (Table S1) specific to the genes were combined with cDNA (50 ng) for RT-PCR in triplicate using GoTaq® qPCR Master Mix (Promega Corporation).

RNA sequencing

For the RNA sample preparations, 1 μg of total RNA was used as input material for each sample. The first strand of cDNA was synthesized using random hexamer priming. The quality of the library was evaluated by using both the Qubit@ 2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2100 system. The library was then sequenced on an Illumina NovaSeq6000 platform, which generated 150 bp paired-end reads. The read pairs from each sample were aligned to the reference genome (*H. sapiens*, GRCh38) (see Supplementary Methods).

Statistical analysis

All the experiments were performed in triplicates. The data are presented as the mean ± standard deviation. Statistical analyses were performed using two-tailed Student's t-test.

Results

The prevalence of *T. tenax* in patients with lung cancer in our cohort

We obtained 27 bronchoalveolar lavage fluid (BALF) samples, comprising 16 males and 11 females, from patients with lung cancer at NCKUH with the approval of Institutional Review Board. BALF samples were retrieved from 27 patients with lung cancer. Table 1 presented the key demographic characteristics of these patients, the mean age (SD) was 64.4 ± 8.7 years-old, and 16 (59.26%) patients were male, 17 (62.96%) and 8 (29.63%) patients were diagnosed as adenocarcinoma and squamous cell carcinoma, respectively. A majority of the patients fell within the age range of 40—80 years (96.3%). A total of 19 (70.37%) patient was diagnosed as stage IV lung cancer.

To investigate the prevalence of trichomonads in patients with lung cancer, we conducted nucleotide isolation and nested PCR, subsequently confirming species identification. The results revealed an overall positive rate of 29.63% (8/27). Regarding the prevalence of trichomonads, there was no statistically significant difference between patients with different histologic types and stages of lung cancer. Among the eight positive specimens, seven as *Trichomonas tenax* and ne was identified as *Tetratrichomonas* spp. through BLAST tools in the Genebank. Table showed

Table 1 Distribution of Trichomonads detected via nested PCR of BALF samples from patients with lung cancer.

Characteristics	Trichomonads positive	Trichomonads negative	Total
BALF sample n (%)	8 (29.63)	19 (70.37)	27 (100)
Gender			
Male	6 (37.5)	10 (62.5)	16 (100)
Female	2 (18.18)	9 (81.82)	11 (100)
Age			
40—60	3 (30)	7 (70)	10 (100)
61—80	5 (31.25)	11 (68.75)	16 (100)
81↑	0 (0)	1 (100)	1 (100)
Pathological type n (%)			
ADC	5 (29.4)	12 (70.6)	17 (100)
SCC	3 (37.5)	5 (62.5)	8 (100)
SCLC	0 (0)	2 (100)	2 (100)
Stage n (%)			
Stage I	2 (100)	0 (0)	2 (100)
Stage II	0 (0)	1 (100)	1 (100)
Stage III	1 (20)	4 (80)	5 (100)
Stage IV	5 (26.32)	14 (73.68)	19 (100)

that trichomonads was predominantly detected in cases of adenocarcinoma (29.4%) and squamous cell carcinoma (37.5%). In this study, we have successfully unveiled the prevalence of trichomonads in patients diagnosed with lung cancer for the first time.

Impact of *T. tenax* on motility and proliferation in pulmonary cancer cells

In our previous study, we observed that *T. tenax* did not induce cytopathic effects on pulmonary cells; however, it triggered the production of IL-6 [13]. Thus, we aimed to assess whether *T. tenax* could augment the cell growth of a lung cancer cell line through co-incubation experiments. After 24 h of co-culture with *T. tenax* and lung cancer cells, *T. tenax* did not induce damage to the epithelium cells and did not affect their growth (Fig. 1A). However, after an extended 48-h incubation, *T. tenax* substantially promoted the growth of lung cancer cells compared to the non-treated group (Fig. 1B). Transwell assays were performed to further investigate the effect of *T. tenax* on cell migration. *T. tenax* significantly increased the migratory capabilities of NCI—H292 cells compared to the non-treated group (Fig. 1C). These results indicated that *T. tenax* enhances cell proliferation and migration in pulmonary cancer cells in vitro.

Differentially expressed genes of NCI—H292 cells response to *T. tenax* invasion

Based on the results of proliferation and migration assay, we suggested that *T. tenax* might play a role in the cancer progress. Next, we purposed to elucidate the responses of the protozoan in human pulmonary cancer cells by RNA-seq. To our knowledge, this represents the initial utilization of this technology in the human pulmonary cancer cell line to describe the impact of an oral protozoan. A total of 803 genes from 34352 Raw gene counts were identified as DEGs with the thresholds (log2 fold change >1, log10 p.adjust <0.05). The cluster heatmap visually represents the expression profiles of triplicated samples in each group (Fig. 2A). Upon co-incubation *T. tenax* with NCI—H292 cells, 526 genes exhibited up-regulation, while 277 genes demonstrated down-regulation (Fig. 2B). This comprehensive analysis sheds light on the intricate gene expression changes induced by *T. tenax* invasion in pulmonary cancer cells.

T. tenax invasion elicits hypoxia responses in NCI—H292 cells

The gene expression profile of *T. tenax*-infected NCI—H292 cells revealed a up-regulation of hypoxia responses. Among the top 30 Gene Ontology (GO) enrichment, predominantly associated with "biological process (BP)", the majority featured up-regulated DEGs (Fig. 3A). The three most enriched GO terms were "response to decreased oxygen levels", "response to oxygen levels", and "response to hypoxia". The GO enrichment map exhibited the network of the DEGs (Fig. 3B). In addition to the hypoxia responses, the infected cells exhibited up-regulation of DEGs belonged to "regulation of vasculature development", and "positive regulation of vasculature development".

The top enrichment pathways included the "HIF-1 signaling pathway" (hypoxia-inducible factor 1 signaling pathway) (Fig. S1A). The GO analysis highlighted that *T. tenax* infection induced hypoxia-liked stimulus and caused the vasculature development. In the Disease Ontology (DO) analysis, the DEGs infected cells were enriched in the DO terms related to anoxia, pulmonary fibrosis, and tumor (Fig. S1B). The analysis indicated the hypoxia responses and the protist infection in the NCI—H292 cells.

T. tenax enhanced the expression levels of cancer-associated genes

We performed qRT-PCR to evaluate the expression of numerous cancer-associated differentially expressed genes (DEGs). The expression levels of genes associated with cancer, including those related to proliferation and migration (*LUCAT1*, *VEGFC*, *ADAM8*, *EMP1*, *HBEGF*, and *TMPRSS4*), as well as drug sensitivity (*AKR1B1*, and *ABCC3*), were upregulated upon exposure to *T. tenax* (Fig. 4). These qRT-PCR findings were in accordance with the RNA-seq outcomes and also suggested that *T. tenax* upregulated these genes, potentially contributing to cancer progression, and promoting cancer migration and proliferation.

Discussion

In previous study, we first revealed an association between periodontitis and oral microbes and determined the prevalence of *T. tenax* in periodontitis patients in Taiwan.[8] In this study, we revealed the presence of trichomonads in

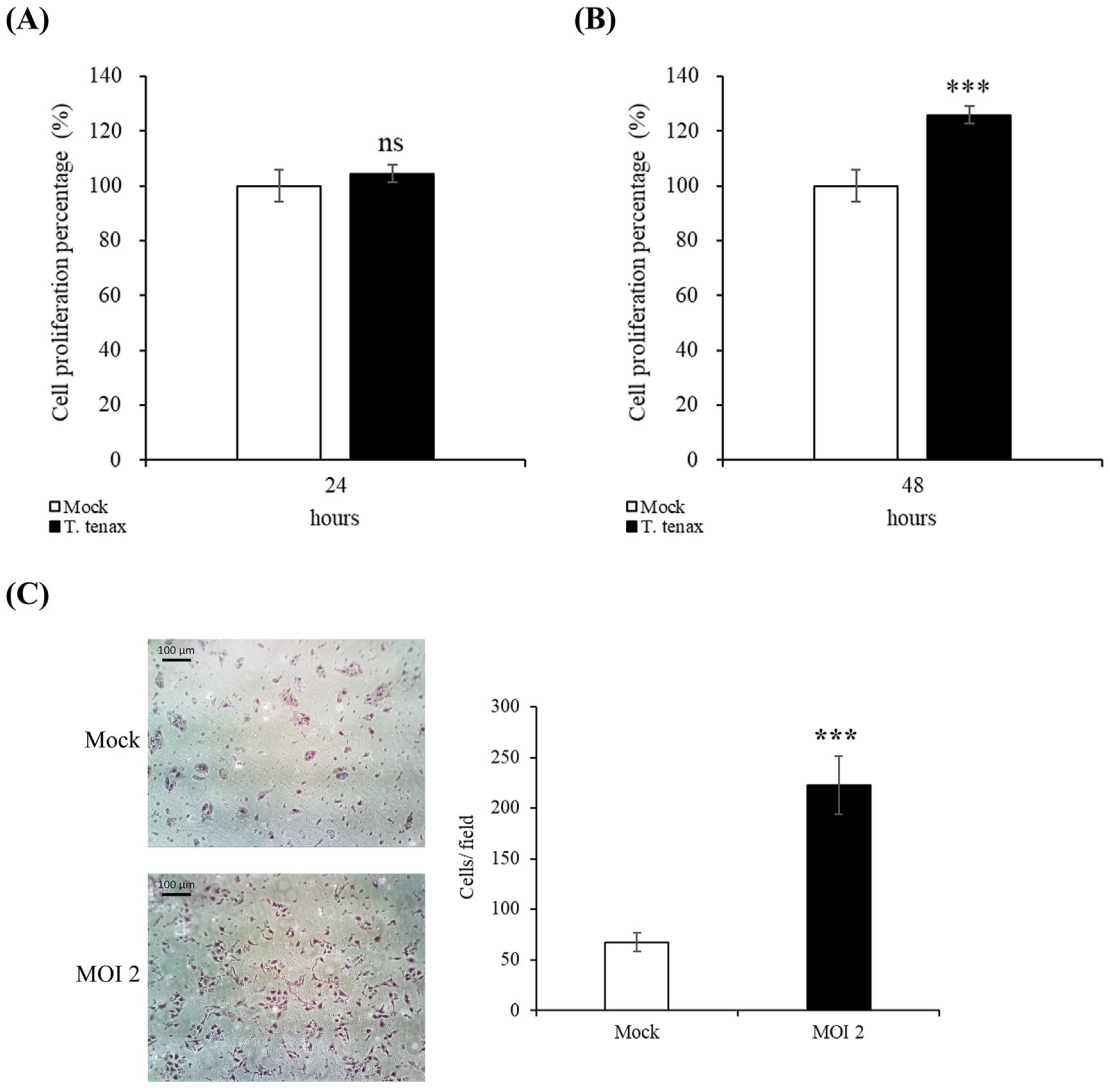

Fig. 1. Cell proliferation, migration, and adhesion assays.
After H292 was treated with or without *T. tenax* for 24 (A) or 48 (B) hours, we measured cell proliferation by using a CCK8 proliferation assay. (C) Transwell assays were also performed to analyze *T. tenax*-induced migration determined by a colony counting assay. The left column (microphotographs) showed the cells that migrated to the lower side of the filter. The quantified number of cells that migrated through the filter in each group was shown in the right column. (***, $p < 0.001$, compared to the control group).

BALF samples from patients with lung cancer in Taiwan through nested PCR methods. Several case reports have identified trichomonads in patients with empyema and/or pleural effusion.[14,15] The oral—lung axis theory postulates that the aspiration of oral secretions into the lungs potentially contributes to the pulmonary diseases.[14,15] Initial investigations unveiled a noteworthy similarity in the oral and lung microbiota, possibly attributed to the anatomical proximity and microaspiration between these sites.[16] Past studies implicated a multitude of anaerobic oral bacteria, including *Fusobacterium nucleatum* and *Prevotella oralis*, in the development of pneumonia, pulmonary abscess, or empyema.[17] This is the first study revealed oral protozoan prevalence in patients with lung cancer in Taiwan.

Microorganisms play a role in the carcinogenic process of cells, influencing processes such as proliferation and migration.[18] Our previous experiments indicated that *T. tenax* did not damage the pulmonary cells but induced their significant production of IL-6 in 24 h.[13] IL-6 affects the initial stages of tumor development through cell growth and movement.[9] We demonstrated that *T. tenax* significantly augmented the cell growth and the migratory capacity of pulmonary cancer cells. The findings provided insight into the host—parasite interaction that pathogens participate in the cancer progress.

Our study found that *T. tenax* enhanced the viability of H292 cells, implying that *T. tenax* may accelerate the cell growth. RNA sequencing results revealed the genetic mechanisms in the responses of NSCLC cell line under the presence of *T. tenax*. The results indicated that *T. tenax* treated H292 cells upregulated *LUCAT1*, which reported to be involved in smoking-related lung cancer.[19] Clinical reports also revealed that advanced stages IIIB and IV adenocarcinomas commonly express higher levels of ADAM8,

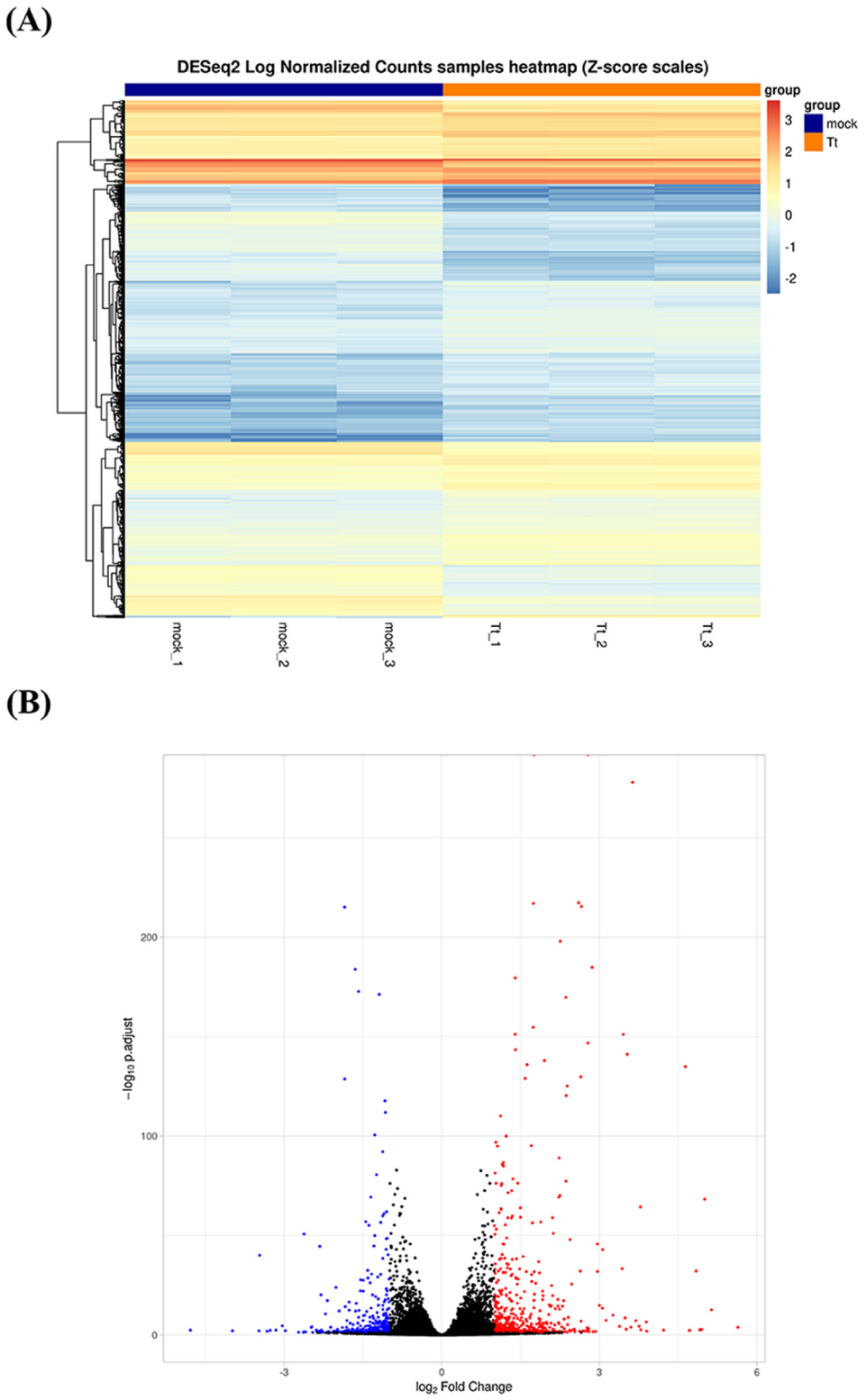

Fig. 2. DEGs between mock and Tt-infected groups.
(A) The cluster heatmap of DEGs. The X-axis is the samples of each group (mock and Tt-infected groups) and the Y-axis is the genes of the samples. Red indicates high expression levels and blue color represents low expression levels. (B) The volcano plot of DEGs The DEGs were identified with the thresholds (log2 fold change = 1, log10 p.adjust = 0.05). 526 up-regulated DEGs (red) and 277 down-regulated DEGs (blue). The X-axis is log2 fold change and the Y-axis is -log10 (p.adjust).

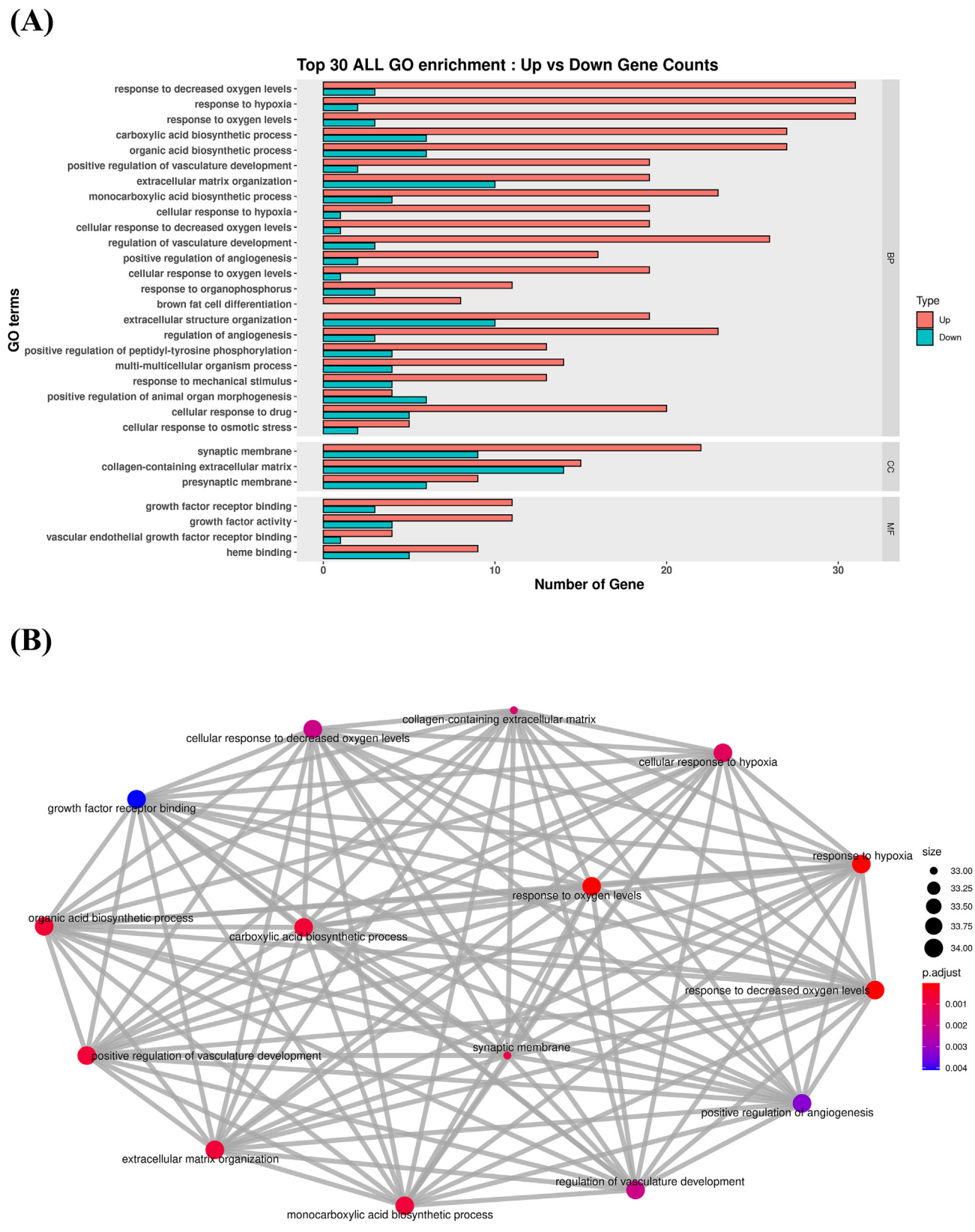

Fig. 3. GO enrichment of DEGs.
(A) The top 30 enriched GO terms of DEGs. The Y-axis is the enriched GO terms and the X-axis is the number of genes. The red bar indicates the up-regulated genes and the blue bar indicates the down-regulated genes. (B) The GO enrichment map. The GO nodes are connected in the diagram if there are common genes between enriched terms. The node color in the network diagram indicates the magnitude of p.adjust, while node size represents the number of genes in the GO terms.

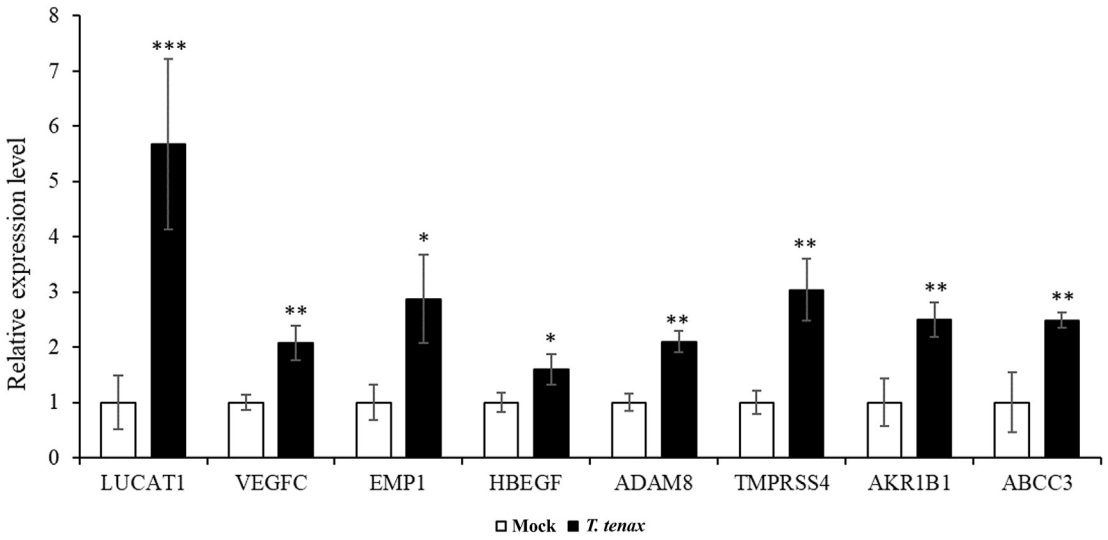

Fig. 4. ***T. tenax*** **induced tumor-related gene expression in H292.**
H292 cells were treated with or without *T. tenax* at at the density of MOI 2 for 24 h, and the relative mRNA expression levels of selected genes were determined with real-time PCR. The fold change was compared to untreated cells. The values are expressed as the means ± SD. (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, compared to the control group).

which was abundantly expressed in most lung cancers, than adenocarcinomas at stages I-IIIA.[20] Studies have indicated that the EMP family members are expressed in various cancers and reveal their role in oncogenesis and tumor progression through activate the PI3K/AKT signaling pathway.[21] TMPRSS4 presents in several lung and colon cancer cell lines.[22] Additionally, HBEGF which is a member of the epidermal growth factor (EGF) family ligands drives arsenic-induced lung cancer development through the activation of the ERK/HIF-1α/VEGF pathway.[23] One of the dominant pathways for cancer cells to adapt to the harsh hypoxic microenvironment is the activation of HIF-1 transcription factor.[24] These genes are involved in regulating crucial biological processes that participate in glucose metabolism, cell proliferation, migration, and angiogenesis.[24] Among the cell responses to *T. tenax* invasion, the enriched GO terms were related to oxygen levels and hypoxia. We suggested that treatment with *T. tenax* induced pulmonary cell stress, subsequently activating hypoxia-associated responses in H292 cells.

Multidrug resistance is one of the risks that contributes to the failure of chemotherapy in NSCLC.[25,26] The suppression of AKR1B1 restored sensitivity to EGFR TKIs in patient-derived xenograft tumors.[25] One of the reasons for multidrug resistance is the overexpression of ATP-binding cassette (ABC) transporters which function as a pump to decrease the intracellular drug concentrations.[26] Our RNA-seq analyses and qPCR validation demonstrated *T. tenax* up-regulated the expression of *AKR1B1* and *ABCC3*. The *T. tenax*-induced stress not only up-regulated the signal of proliferation and migration but also potentially decreased the sensitivity to drug treatment.

In summary, our research identified the presence of *T. tenax* in lung cancer samples. Our in vitro assays examining migration and proliferation patterns unveiled the responses of lung cancer cells to *T. tenax*-induced stress. Further analysis through RNA-seq and qRT-PCR indicated that the stress induced by *T. tenax* potentially drives tumor progression by upregulating genes associated with inflammation, migration, proliferation, and drug resistance. Thus, in addition to bacteria associated with periodontitis, our findings suggest that oral pathogenic protozoa may exacerbate pulmonary symptoms by traveling through the respiratory tract.

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CRediT authorship contribution statement

Chun-Hsien Chen: Data curation, Formal analysis, Writing — original draft, Writing — review & editing. **Chin-Wei Kuo:** Data curation, Formal analysis, Funding acquisition, Resources, Writing — original draft. **Chih-Ming Tsai:** Formal analysis, Methodology, Software, Visualization. **Zih-Bin Hong:** Data curation, Formal analysis, Methodology, Validation. **Ching-Han Lai:** Funding acquisition, Project administration, Resources, Supervision, Writing — review & editing. **Tzu-Yi Chan:** Data curation, Formal analysis. **Wei-Chen Lin:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Validation, Writing — original draft, Writing — review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021;**71**(3):209—49.
2. Chien LH, Jiang HF, Tsai FY, Chang HY, Freedman ND, Rothman N, et al. Incidence of lung adenocarcinoma by age, sex, and smoking status in taiwan. *JAMA Netw Open* 2023;**6**(11):e2340704.
3. Guinane CM, Cotter PD. Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. *Therap Adv Gastroenterol* 2013;**6**(4):295—308.
4. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, et al. A review of human carcinogens–Part B: biological agents. *Lancet Oncol* 2009;**10**(4):321—2.
5. van Tong H, Brindley PJ, Meyer CG, Velavan TP. Parasite infection, carcinogenesis and human malignancy. *EBioMedicine* 2017;**15**:12—23.
6. Mallat H, Podglajen I, Lavarde V, Mainardi JL, Frappier J, Cornet M. Molecular characterization of Trichomonas tenax causing pulmonary infection. *J Clin Microbiol* 2004;**42**(8):3886—7.
7. Shigdel R, Johannessen A, Lin H, Peddada S, Gomez Real F, Ringel-Kulka T, et al. Oral bacterial composition associated with lung function and lung inflammation in a community-based Norwegian population. *Respir Res* 2023;**24**(1):183.
8. Chen YC, Huang JM, Chang YY, Yuan K, Lin WC, Ting CC. Comparative prevalence of oral bacteria and protozoa in patients with periodontitis in Taiwan. *Oral Dis* 2023:1—5.
9. Murata D, Azuma K, Murotani K, Matsuo N, Matama G, Tokito T, et al. Survival and soluble immune mediators of immune checkpoint inhibitor-induced interstitial lung disease in patients with non-small cell lung cancer. *Lung Cancer* 2023;**184**:107351.
10. Meyer KC, Raghu G, Baughman RP, Brown KK, Costabel U, du Bois RM, et al. An official American Thoracic Society clinical practice guideline: the clinical utility of bronchoalveolar lavage cellular analysis in interstitial lung disease. *Am J Respir Crit Care Med* 2012;**185**(9):1004—14.
11. Lin C, Ying F, Lai Y, Li X, Xue X, Zhou T, et al. Use of nested PCR for the detection of trichomonads in bronchoalveolar lavage fluid. *BMC Infect Dis* 2019;**19**(1):512.
12. Justus CR, Leffler N, Ruiz-Echevarria M, Yang LV. In vitro cell migration and invasion assays. *J Vis Exp* 2014;(88).
13. Hong ZB, Lai YT, Chen CH, Chen YJ, Chen CC, Lin WC. Trichomonas tenax induces barrier defects and modulates the inflammatory cytotoxicity of gingival and pulmonary epithelial cells. *Parasite* 2023;**30**:7.
14. Wang HK, Jerng JS, Su KE, Chang SC, Yang PC. Trichomonas empyema with respiratory failure. *Am J Trop Med Hyg* 2006;**75**(6):1234—6.
15. Wu Y, Ye Y, Yang Y, Yang W, Lin J, Cao K. Pyopneumothorax from coinfection by Trichomonas tenax and Geotrichum capitatum in a child from China: a case report. *BMC Infect Dis* 2021;**21**(1):842.
16. Gaeckle NT, Pragman AA, Pendleton KM, Baldomero AK, Criner GJ. The oral-lung Axis: the impact of oral health on lung health. *Respir Care* 2020;**65**(8):1211—20.
17. Pathak JL, Yan Y, Zhang Q, Wang L, Ge L. The role of oral microbiome in respiratory health and diseases. *Respir Med* 2021;**185**:106475.
18. Kamangar F, Dawsey SM, Blaser MJ, Perez-Perez GI, Pietinen P, Newschaffer CJ, et al. Opposing risks of gastric cardia and noncardia gastric adenocarcinomas associated with Helicobacter pylori seropositivity. *J Natl Cancer Inst* 2006;**98**(20):1445—52.
19. Thai P, Statt S, Chen CH, Liang E, Campbell C, Wu R. Characterization of a novel long noncoding RNA, SCAL1, induced by cigarette smoke and elevated in lung cancer cell lines. *Am J Respir Cell Mol Biol* 2013;**49**(2):204—11.
20. Ishikawa N, Daigo Y, Yasui W, Inai K, Nishimura H, Tsuchiya E, et al. ADAM8 as a novel serological and histochemical marker for lung cancer. *Clin Cancer Res* 2004;**10**(24):8363—70.
21. Miao L, Jiang Z, Wang J, Yang N, Qi Q, Zhou W, et al. Epithelial membrane protein 1 promotes glioblastoma progression through the PI3K/AKT/mTOR signaling pathway. *Oncol Rep* 2019;**42**(2):605—14.
22. Jung H, Lee KP, Park SJ, Park JH, Jang YS, Choi SY, et al. TMPRSS4 promotes invasion, migration and metastasis of human tumor cells by facilitating an epithelial-mesenchymal transition. *Oncogene* 2008;**27**(18):2635—47.
23. Wang L, Lu YF, Wang CS, Xie YX, Zhao YQ, Qian YC, et al. HB-EGF activates the EGFR/HIF-1 alpha pathway to induce proliferation of arsenic-transformed cells and tumor growth. *Front Oncol* 2020;**10**:1019.
24. Semenza GL. HIF-1 and mechanisms of hypoxia sensing. *Curr Opin Cell Biol* 2001;**13**(2):167—71.
25. Zhang KR, Zhang YF, Lei HM, Tang YB, Ma CS, Lv QM, et al. Targeting AKR1B1 inhibits glutathione de novo synthesis to overcome acquired resistance to EGFR-targeted therapy in lung cancer. *Sci Transl Med* 2021;**13**(614):eabg6428.
26. Liu L, Liu N, Liu B, Yang Y, Zhang Q, Zhang W, et al. Are circulating autoantibodies to ABCC3 transporter a potential biomarker for lung cancer? *J Cancer Res Clin Oncol* 2012;**138**(10):1737—42.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.05.001.