Characteristics and clinical significance of polyploid giant cancer cells in laryngeal carcinoma

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Abstract
Objectives: We aimed to construct an induction system for polyploid giant cancer cells (PGCCs), as well as to investigate PGCC features and clinical significance.

Methods: A laryngeal neoplasm-PGCC induction system was constructed using paclitaxel liposomes (PTX). We used western blots to compare expression of epithelial-mesenchymal transition-related proteins, stem cell interrelated proteins, and cyclin-associated proteins. We then measured PGCC count in tissue samples of patients with laryngeal neoplasms and analyzed its relationship with prognosis. Statistical significance was determined using t-tests.

Results: PTX successfully induced PGCCs. Western blotting showed that CyclinB1, CDC25C, CDK1, E-cadherin, and EIF-4A expression decreased in PGCCs compared with normal cancer cells, whereas vimentin and CD133 expression increased. Number of PGCCs in laryngeal cancer tissues and overall survival time were inversely correlated (P < .05).

Conclusions: PTX successfully induces PGCC formation in laryngeal carcinoma, which may be the cause of poor prognosis in patients with laryngeal cancer.

Level of Evidence: 4.

KEYWORDS
epithelial mesenchymal transformation, laryngeal neoplasms, polyploid giant cancer cells, prognosis

1 | INTRODUCTION

Laryngeal cancer has a high incidence, accounting for 26% of malignant head and neck tumors worldwide, of which 95% are squamous cell carcinomas. Although early diagnosis is straightforward, relapse and tumor metastasis can still occur after treatment, with a 5-year survival rate of only 50%. Therefore, exploring the specific mechanisms of recurrence and metastasis in laryngeal cancer patients is of great importance for improving prognosis.

Available research indicates that polyploid giant cancer cells (PGCCs) are important factors in tumorigenesis, invasion, development, metastasis, and chemotheray resistance. This subgroup has characteristics of cancer stem cells and contain either a large nucleus or multiple nuclei. Previously, PGCCs were thought to be...
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2  | MATERIALS AND METHODS

The study protocol was approved by the Ethics Committee for Human Experiments of the Affiliated Hospital of Nantong University (2020-L160). Informed consent was obtained from all patients.

2.1  | Cell culture

Human laryngeal tumor cell line TU212 was obtained from the Laboratory of Medical College of Shanghai Jiao-tong University. Cells were cultured in IMDM medium containing 10% FBS and 1% penicillin-streptomycin, using an incubator maintained at 5% CO₂ and 37°C.

2.2  | Induction of laryngeal cancer cell-PGCCs

TU212 was inoculated in a cell culture dish and cultured until cell fusion reached 80% to 90%. After 16 hours of induction with a base culture of PTX (Paclitaxel liposome for injection, China, 0.04 mg/mL), cells were gently rinsed using phosphate buffered saline (PBS). Culturing continued in a complete medium, with the solution changing every 2 days. Approximately 20 to 25 days after PTX removal, ordinary Tu212 cells experienced 100% mortality, and only PGCCs survived. Surviving PGCCs were collected for follow-up experiments.

2.3  | Immunofluorescence

TU212 cells were cultured on glass coverslips. After rinsing with fresh PBS for 20 minutes, cells were fixed using 4% paraformaldehyde at room temperature. Next, they were blocked in 4% BSA for 2 hours at 4°C, followed by overnight incubation with primary antibody against vimentin (Cell Signaling Technology, USA, 5741; dilution ratio: 1:100) at 4°C. Cells were then washed with fresh 1× PBS for 15 minutes and incubated in a solution of Alexa Fluor 488 (Beyotime, China, A0423; dilution ratio: 1:200). After three PBS washes and staining with DAPI (Invitrogen, D1306) for 15 minutes, samples were observed under a fluorescence microscope (Zeiss, Germany).

2.4  | Live/dead assay

Live/dead fluorescent staining was performed using a LIVE/DEAD assay kit (BestBio, China, BB-4101-1). Calcein AM emits green fluorescence in living cells, whereas propidium iodide causes red fluorescence in dead cells. First, PGCCs were cultured in cell culture dishes, then rinsed twice with PBS before using the assay kit. Subsequently, they were incubated for 25 minutes. Samples were observed under a fluorescence microscope (Zeiss, Germany).

2.5  | Western blotting

Three groups of cells were used: control (normal diploid laryngeal cancer cells), drug-withdrawal (PTX removed), and PGCC. After extraction using RIPA buffer with protease inhibitors, protein concentration was measured with BCA and electrophoresis using a 6% to 10% SDS-PAGE gel. Proteins were then transferred onto PVDF membranes and blocked with 5% non-fat milk powder for 1 hour. Membranes were incubated with primary antibodies overnight at 4°C, washed three times with TBST, incubated with secondary antibody for 1 hour, rewashed, and subjected to the ECL kit (Millipore, USA, WBKLS0500). Resultant signals were exposed to X-ray film for development and scanned into a computer using radiographic film. Antibodies used were β-Actin (Proteintech, Wuhan, China, 66009-1-1g, dilution ratio: 1:600), CDC25C (Proteintech, Wuhan, China, 16485-1-AP; dilution ratio: 1:1000), CDK1 (Proteintech, Wuhan, China, 19532-1-AP; dilution ratio: 1:1000), CyclinB1 (Proteintech, Wuhan, China, 55004-1-AP; dilution ratio: 1:1000), and CD133 (Proteintech, Wuhan, China, 18470-1-AP; dilution ratio: 1:1000). E-cadherin (Cell Signaling Technology, USA, 3195; dilution ratio: 1:2000), and vimentin (Cell Signaling Technology, USA, 5741; dilution ratio: 1:2000).
FIGURE 1  PTX treatment can induce PGCC formation. (A) After PTX treatment for 16 hours and at 20 days posttreatment, TU212 was induced to form PGCCs. (B) (a) Normal TU212 without PTX treatment. (b) Cell morphology was observed after 16 hours of PTX treatment. (c,d) After 20 days, giant nuclei developed. Scale: 20 μm. (C) Cytoplasm was stained with vimentin (green) and nuclei were stained with Hoechst (blue). (a-c) Control group. (d-i) Multicore PGCCs; red arrow indicates nucleus. Scale: 20 μm. (D) (a-d) Budding PGCCs, with DNA present in PGCC branches. Red arrow indicates nucleus. Scale: 20 μm. (E) PGCCs were alive. Images taken under 400× microscope.
2.6 | Detection of PGCCs in pathological sections

Tissue samples from 102 patients diagnosed with laryngeal carcinoma during 2013 to 2020 were collected and stored at the Department of Surgery and Pathology, Affiliated Hospital of Nantong University. Hematoxylin-eosin (HE)-stained sections from each sample were subjected to a count of PGCCs by three independent senior pathologists. For counting, nine fields of view were randomly selected for each slice under a 200-fold microscope (Zeiss, Germany).

2.7 | Statistical analyses

Data were analyzed using t-tests in SPSS 19.0. Kaplan-Meier survival curves were used to express survival results in GraphPad Prism 8.0 (GraphPad Prism Software, Inc). Statistical significance was defined as \( P < .05 \).

3 | RESULTS

3.1 | PTX treatment can induce PGCC formation

To investigate the influence of PTX treatment on laryngeal carcinoma, we first treated TU212 cells with PTX for 16 hours (Figure 1A). Microscopic observation revealed a few TU212 cells that became round and bright when PTX was withdrawn (Figure 1B-a,B-b). At 20 days posttreatment, most normal-sized diploid cells were killed, and only a minority of PGCCs survived. These PGCCs were multinucleated and much larger than normal TU212 cells (Figure 1B-c,
B-d). Normal Tu212 cells were around 50 μm and grew adherent to the wall, with a polygonal shape and clear outline (Figure 1B). In contrast, PGCCs were irregular and about 160 μm in size, with stagnant proliferation. We then investigated changes to nucleocytoplasmic distribution after PTX treatment. After labeling chromosomes with DAPI (blue) and cytoplasm with vimentin (green), immunofluorescence identified different nucleus sizes in PGCCs (Figure 1C). In addition, we observed one large nucleus and two small nuclei in the same cell. This asymmetrical division indicates failed mitosis, and could also be the start of splitting genetic material before the daughter cell buds.

At 20 to 25 days post-PTX treatment, daughter cells budded from surviving PGCCs (Figure 1D), specifically through extending long branches containing DNA. Daughter cell nuclei are derived from PGCC nuclei via these branches, consistent with our observations. In addition, we confirmed that PGCCs were alive using live/dead cell double-staining (Figure 1E).

3.2 | Characteristics of PGCCs

We found that PGCCs have the capability to form colonies, also known as the intrinsic of stem cells. We used western blotting to measure changes in stemness. Compared with control TU212, CD133 expression was significantly higher in PGCCs (Figure 2A), suggesting that PGCCs present cancer stem-like cell characteristics.

Because PTX acts on tubulin to stabilize microtubules and cause cell cycle arrest, we investigated expression of cell cycle-related proteins. CDC25C, CDK1, and cyclinB1 expression was lower in PGCCs than in control TU212. CDC25C expression also decreased upon PTX withdrawal. Both control TU212 and PTX-withdrawal groups had lower EIF-4A expression (Figure 2A,B).

Epithelial-to-mesenchymal transition (EMT) refers to the loss of intercellular adhesion in epithelial cells, followed by acquiring the ability to migrate and invade. Some studies have shown that EMT promotes tumor invasion and metastasis. To investigate the complex biological behaviors of PGCCs, we detected protein cleavage with immunoblotting (Figure 2C,D). Decreased E-cadherin expression and increased vimentin expression in PGCCs suggested that EMT had occurred.

3.3 | Clinical significance of PGCC expression in laryngeal carcinoma specimens

Examination of patient tissue specimens revealed PGCCs in pathologic sections, based on known morphological features (Figure 3A).
HE staining revealed significant correlations between PGCC expression and histological differentiation (Figure 3B). Concurrently, we followed up on overall survival times (Table 1) and analyzed them using Kaplan-Meier plots. Number of PGCCs and prognosis time were inversely correlated (Figure 3C-a). Additionally, patients with a high PGCC count had relatively poor prognosis. Thus, PGCCs may be an element indicating poor prognosis in patients with laryngeal carcinoma. In addition, we counted the 3-year survival rate of 51 patients with laryngeal cancer from 2013 to 2016 (Table 2), and demonstrated that those who developed lymph node metastasis had a poor prognosis (\(P < .05\)) (Figure 3C-b).

### 4 | DISCUSSION

Paclitaxel, docetaxel, CoCl\textsubscript{2}, radiotherapy and targeted-therapy agents can induce PGCC formation from tumor diploid cells.\textsuperscript{3,12–15} Clinically, PTX is commonly used for chemotherapy in patients who suffer from laryngeal carcinoma, with greatly improved prognosis. However, some patients still develop “explosive” recurrence and distant metastasis, accompanied by poor prognosis.\textsuperscript{7,12} Therefore, we need to understand the mechanism of this poor prognosis after PTX treatment.

First, we performed long-term observation of TU212 cells after PTX treatment and found that a small number of them did not die, instead generating giant nuclei or multinucleated PGCCs. These cells then generate daughter cells through budding out of branch-like structures. PGCCs are thought to arise from failed mitosis, in a process called endoreplication.\textsuperscript{16} During the normal cancer cell cycle, CDC25C activates CDK1. Subsequently, the binding and translocation of CDK1 and cyclin B1 to the nucleus triggers the G2/M transition of cell cycle.\textsuperscript{17,18} Here, we found that upon PTX withdrawal, CDC25C expression decreased, suggesting a block in cell cycle initiation. Meanwhile, CDC25C, CDK1, and cyclinB1 expression was lower in PGCCs than in control TU212, possibly accounting for this mitotic block and causing the formation of multiple giant nuclei in PGCCs.\textsuperscript{19,20}

We also discovered that PGCCs had significantly lower growth rates than normal TU212 cells. We showed that EIF-4A1 expression decreased in PGCCs. EIF4A is a dead-box helicase that is a part of the translation initiation complex, and lowered EIF-4A1 expression inhibits cell proliferation ability.\textsuperscript{21} Our findings suggested that PTX caused stress in TU212 cells, shutting down protein translation. Upon PTX withdrawal, the cell cycle progressed, but more slowly.

Studies have indicated that PGCCs can express CD133,\textsuperscript{22} a marker of cancer stem cells. CD133 regulates tumorigenesis, self-renewal, angiogenesis, and chemoradiotherapy resistance. Here, we confirmed that CD133 expression is significantly elevated in PGCCs.

Some scholars suggest that PGCCs have a mesenchymal phenotype, meaning a stronger ability to migrate and invade than common diploid cancer cells.\textsuperscript{3,23} Here, we examined the expression of two EMT-associated proteins in PGCCs and TU212 cells without PTX treatment. The first protein is E-cadherin, which promotes epithelial cell adhesion and is widely recognized as a molecular marker of EMT in epithelial tissues.\textsuperscript{24} The second protein is vimentin, an essential part of the cytoskeleton (distributed widely in mesenchymal cells).\textsuperscript{25} We found that PGCCs had lower E-cadherin and higher vimentin.

### TABLE 1

| Parameters                  | Total (102) | PGCCs high expression (22) | PGCCs low expression (80) | \(P\)-value |
|-----------------------------|-------------|---------------------------|---------------------------|-------------|
| Age (years)                 |             |                           |                           | .22         |
| \(\leq 63\)                 | 53          | 8                         | 45                        |             |
| >63                         | 49          | 14                        | 35                        |             |
| Gender                      |             |                           |                           | .06         |
| Male                        | 102         | 22                        | 80                        |             |
| Female                      | 0           | 0                         | 0                         |             |
| Lymph node metastasis       |             |                           |                           | .0001       |
| Positive                    | 22          | 8                         | 14                        |             |
| Negative                    | 80          | 14                        | 66                        |             |
| Prognosis                   |             |                           |                           |             |
| Alive                       | 80          | 9                         | 71                        |             |
| Dead                        | 22          | 13                        | 9                         |             |

### TABLE 2

| Parameters                  | Total (51) | Lymph node metastasis | Lymph node metastasis | \(P\)-value |
|-----------------------------|------------|-----------------------|-----------------------|-------------|
| Prognosis                   |            |                       |                       | .0009       |
| Alive                       | 51         | 12                    | 24                    |             |
| Dead                        | 9          | 2                     | 6                     |             |
expression than normal cancer cells, indicating greater migratory capacity in PGCCs. This trait may be responsible for the development of distant metastases years after treatment.

On this basis, we collected clinical data and counted PGCCs in pathological sections of patients with laryngeal cancer over a period of nearly 7 years. Our results suggest that the number of PGCCs is related to 3-year survival. Furthermore, PGCCs appear to be a poor prognostic factor for patients.

In conclusion, our study demonstrated that a fraction of laryngeal carcinoma cells generate PGCCs after PTX treatment. This occurrence is likely an extremely significant reason for posttreatment tumor recurrence and distant metastasis. Therefore, PGCCs may be a novel therapeutic target for the treatment of laryngeal cancer. Prevention of PGCC formation, as well as exploring the molecular mechanisms of their behaviors, will benefit the refinement of laryngeal cancer therapy.

CONFLICT OF INTEREST
The authors declare no potential conflict of interests.

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REFERENCES
1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018;68:7-30.
2. Forastiere AA, Ismaila N, Lewin JS, et al. Use of larynx-preservation strategies in the treatment of laryngeal cancer: American Society of Clinical Oncology clinical practice guideline update. J Clin Oncol. 2018;36:1143-1169.
3. Niu N, Mercado-Uribe I, Liu J. Dedifferentiation into blastomere-like cancer stem cells via formation of polyploid giant cancer cells. Oncogene. 2017;36:4887-4900.
4. Amend SR, Torga G, Lin KC, et al. Polyploid giant cancer cells: unrecognized actuators of tumorigenesis, metastasis, and resistance. Prostate. 2019;79:1489-1497.
5. Fei F, Zhang D, Yang Z, et al. The number of polyploid giant cancer cells and epithelial-mesenchymal transition-related proteins are associated with invasion and metastasis in human breast cancer. J Exp Clin Cancer Res. 2015;34:158.
6. Zhang S, Mercado-Uribe I, Xing Z, Sun B, Kuang J, Liu J. Generation of cancer stem-like cells through the formation of polyploid giant cancer cells. Oncogene. 2014;33:116-128.
7. Zhang S, Mercado-Uribe I, Liu J. Tumor stroma and differentiated cancer cells can be originated directly from polyploid giant cancer cells induced by paclitaxel. Int J Cancer. 2014;134:508-518.
8. Zhang S, Mercado-Uribe I, Liu J. Generation of erythroid cells from fibroblasts and cancer cells in vitro and in vivo. Cancer Lett. 2013;333:205-212.
9. Mirzayans R, Andrais B, Murray D. Roles of polyploid/multinucleated giant cancer cells in metastasis and disease relapse following anticancer treatment. Cancers. 2018;10:118. https://doi.org/10.3390/cancers10040118
10. Chen J, Niu N, Zhang J, et al. Polyploid giant cancer cells (PGCCs): the evil roots of cancer. Curr Cancer Drug Targets. 2019;19:360-367.
11. Zhang L, Ding P, Lv H, et al. Number of polyploid giant cancer cells and expression of EZH2 are associated with VM formation and tumor grade in human ovarian tumor. Biomed Res Int. 2014;2014:903542.
12. Mittal K, Donthamsetty S, Kaur R, et al. Multinucleated polyploidy drives resistance to Docetaxel chemotherapy in prostate cancer. Br J Cancer. 2017:116:1186-1194.
13. Lopez-Sanchez LM, Jimenez C, Valverde A, et al. CoCi2, a mimic of hypoxia, induces formation of polyploid giant cells with stem characteristics in colon cancer. PLoS One. 2014;9:e99143.
14. White-Gilbertson S, Lu P, Jones CM, et al. Tamoxifen is a candidate first-in-class inhibitor of acid ceramidase that reduces amitotic division in polyploid giant cancer cells—Unrecognized players in tumorigenesis. Cancer Medicine. 2020;9:3142-3152. https://doi.org/10.1002/cam4.2960
15. Tagai V, Roth MG. Loss of Aurora Kinase Signaling Allows Lung Cancer Cells to Adopt Endoreplication and Form Polyploid Giant Cancer Cells That Resist Antimitotic Drugs. Cancer Research. 2021;81:400-413. https://doi.org/10.1158/0008-5472.can-20-1693
16. Shu Z, Row S, Deng WM. Endoreplication: the good, the bad, and the ugly. Trends Cell Biol. 2018;28:465-474.
17. Huang G, Meng L, Tsai RYL. p53 Configures the G2/M arrest response of nucleostemin-deficient cells. Cell Death Discovery. 2015;1: https://doi.org/10.1038/cddiscovery.2015.60
18. Lv H, Shi Y, Zhang L, et al. Polyploid giant cancer cells with budding and the expression of cyclin E, S-phase kinase-associated protein 2, stathmin associated with the grading and metastasis in serous ovarian tumor. BMC Cancer. 2014;14:576.
19. Fei F, Qu J, Liu K, et al. The subcellular location of cyclin B1 and CDC25 associated with the formation of polyploid giant cancer cells and their clinicopathological significance. Lab Invest. 2019;99:483-498.
20. Liu K, Lu R, Zhao Q, et al. Association and clinicopathologic significance of p38MAPK-ERK-JNK-CDC25C with polyploid giant cancer cell formation. Med Oncol. 2019;37:6.
21. Harms U, Andreou AZ, Gubaev A, Klostermeier D. eIF4B, eIF4G and RNA regulate eIF4A activity in translation initiation by modulating the eIF4A conformational cycle. Nucleic Acids Res. 2014;42:7911-7922.
22. Jiang Q, Zhang Q, Wang S, et al. A fraction of CD133+ CNE2 cells is made of giant cancer cells with morphological evidence of asymmetric mitosis. J Cancer. 2015;6:1236-1244.
23. Wang X, Zhang M, Fei F, et al. EMT-related protein expression in polyploid giant cancer cells and their daughter cells with different passages after triptolide treatment. Med Oncol. 2019;36:82.
24. Derksen PWB, Liu X, Saridin F, et al. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. Cancer Cell. 2006;10:437-449.
25. Yao X, Wang X, Wang Z, et al. Clinicopathological and prognostic significance of epithelial mesenchymal transition-related protein expression in intrahepatic cholangiocarcinoma. Onco Targets Ther. 2012;5:255-261.

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