



Polyploid Giant Cancer Cells are Involved in Paclitaxel-induced Chemotherapy Resistance via Neosis in Non-Small Cell Lung Cancer

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Abstract

Paclitaxel is widely used to treat cancer patients through the blocking of mitosis, yet results in formation of polyploidy giant cancer cells (PGCCs), which are generally believed to be non-dividing cells and involve in tumor recurrence in certain cancers, such as ovarian cancer, breast cancer and colorectal cancer, et al. However, there are few studies related to PGCCs in lung cancer. Herein, we observed PGCCs in tumor tissues of non-small cell lung cancer (NSCLC) patients and verified that paclitaxel could induce PGCCs in NSCLC cells *in vitro*. PGCCs was revealed to bud via neosis to form daughter cells, which have stronger ability of migration, proliferation and colony formation. PGCCs were also found to be not only associated with tumor heterogeneity but also contribute to paclitaxel resistance. These results suggested that PGCCs were closely related to chemotherapy resistance to paclitaxel, leading to tumor recurrence in NSCLC patients.

Keywords: Polyploid giant cancer cells; Neosis; Paclitaxel; Chemotherapy resistance; Non-small cell lung cancer

Introduction

Lung cancer ranks globally as one of the most frequently diagnosed cancers and is the leading cause of cancer-related deaths for many years, representing approximately 11.4% of cancers diagnosed and 18.0% of deaths worldwide in 2020, of which non-small cell lung cancer (NSCLC) accounts for approximately 85% [1-3]. A number of important advancements in NSCLC treatment, such as small molecule tyrosine kinase inhibitors and immunotherapy, have been achieved over the past two decades. However, the overall cure and survival of NSCLC patients remain low, with a 5-year survival rate of less than 15% in most countries [3,4]. As known, NSCLC is a heterogeneous disease, the therapeutic responses to current standard treatments are poor except for the localized cancers. Tumor metastasis and recurrence are still the main causes of death for advanced NSCLC, and remain unresolved [5].

Paclitaxel (PTX), a classic small molecule inhibitor of cell mitosis, has held a first-line chemotherapy agent for advanced NSCLC patients for many years, even new therapeutic modalities have been introduced and recommended in recent years. However, PTX resistance remains an outstanding issue although diverse mechanisms of PTX resistance have been well elucidated, such as alternations to tubulin structures, overexpression of ABC transporters and epithelial-mesenchymal transition, etc [6-9]. As noted, the microtubule stabilizing agent PTX would induce polyploidy giant cancer cells (PGCCs) through blocking of mitosis, causing breast cancer cells to become more resistant to paclitaxel [10]. Actually, as a special subgroup of malignant tumor cells that are at least three times larger than conventional tumor cells, have more than three non-conventional nuclei in morphology and produce daughter cells through asymmetric cell division, PGCCs have aroused more and more interests and attentions in recent years [11]. PGCCs were also observed in colorectal cancer [12], ovarian cancer [13], breast cancer [14] and prostate cancer [15], etc., which are thought to be blastomere-like cancer stem cells and closely associated with tumorigenesis, drug resistance and

metastasis [11,16]. However, few studies have focused on PGCCs in lung cancer.

Neosis is a novel manner of cell division which was first reported by Sundaram et al. [17]. Some surviving PGCCs can undergo neosis via asymmetrically intracellular cytokinesis, nuclear budding to produce some mononuclear cells [17]. The cells via neosis were considered important in tumor self-renewal [18-20] and tumor therapeutic resistance [21, 22] due to their stem cell characteristics [23-25]. Also, the studies about neosis focus on colorectal cancer, ovarian cancer, breast cancer and prostate cancer, few studies have focused on PGCCs in lung cancer.

Herein we observed PGCCs in tumor tissues of NSCLC patients, and further explored the biological behaviors of PGCCs in non-small cell lung cancer cells upon paclitaxel treatment. Our study revealed that PGCCs were closely related to chemotherapy resistance to paclitaxel, leading to tumor recurrence in NSCLC patients.

Materials and methods

Cancer cell lines and tumor tissue samples

Human lung cancer cell lines A549 (ATCC® CCL-185™), H1299 (ATCC® CRL-5803™), and PC-9 (ECACC 90071810) were obtained from American Tissue Culture Collection and European Collection of Cell Cultures. Cells were cultured with RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) plus 100 U/ml penicillin (Gibco, NY, USA) and 100 µg/ml streptomycin (Gibco, NY, USA) in a humidified atmosphere of 5% CO₂ at 37 °C.

Paraffin-embedded non-small cell lung cancer tissue samples (n = 47) were obtained from the Clinical BioBank of Shanghai Chest Hospital. All patients were histologically diagnosed and no patients were treated prior to surgical tumor resection. Pathologically, 12 tissue samples were high-grade lung cancer, 25 were intermediate, and 10 were low. The study was approved by the Ethical Review Board of Shanghai Chest Hospital, and the patient information was kept confidential.

Hematoxylin and eosin (H&E) staining

4 mm sections from formalin-fixed, paraffin-embedded lung cancer tissues were deparaffinized, rehydrated, and counterstained with hematoxylin for 1 min and eosin for 2 min. The sections were then dehydrated and mounted on coverslips. The morphology of tissue samples was observed and photographed under a microscope (ZEISS, AxioLab A1, Jena, Germany).

Induction of PGCCs

When the confluence of A549, H1299 and PC-9 cells reached 80-90%, paclitaxel (Bristol-Myers Squibb, NY, USA) was added at

a final concentration of 75 ng/ml (A549 PC-9), 300 ng/ml (H1299) and cultured for another 48 hrs. The PTX-containing medium was then removed, and cells were washed with PBS (phosphate buffered saline) solution and added with regular medium for further culture for 10-30 days. Most regular-sized of cancer cells would die while a few scattered PGCCs survived after PTX treatment. The surviving PGCCs were observed and photographed under an inverted microscope (ZESSI Primo VertHD, Jena, Germany). Daughter cells were observed to be generated from PGCCs as the recovery time prolonged. When daughter cells reached 90%, they are passaged, expanded and collected for subsequent experiments.

Flow cytometry assay

The lung cancer cells A549, H1299 and PC-9 were cultured upon paclitaxel treatment or not for 48 hrs. Then paclitaxel was removed, and cells were washed with PBS and continued to culture with regular medium for 2 days. 1-2×10⁶ cells were collected by digestion with 0.25 % trypsin (Gibco, NY, USA) and resuspended in 1 ml PBS. 3 ml of absolute ethanol (pre-cooled at -20°C) was added slowly to the resuspended cell with high-speed stir, and further fixed at -20°C overnight. The fixed cells were centrifuged at 400 g for 5 min, discarded the ethanol, resuspended in 2-5 ml of PBS for 15 min to rehydrate. Samples were centrifuged, discarded the supernatant and resuspended in PBS supplemented with 50 mg/ml propidium iodide (BD Biosciences, NJ, USA) and 100 mg/ml RNase A for 30 min incubation. Flow cytometry assay was performed routinely on a FACS Canto II flow cytometer (BD Biosciences, NJ, USA).

Hoechst 33342 staining

Cell slides were paved in a 6-well plate, and the lung cancer cells A549, H1299 and PC-9 were seeded and routinely cultured for 24-48 hrs. When cancer cells reached 80-90% confluence, paclitaxel was added and cultured for another 48 hrs. The PTX-containing medium was then removed, cells were washed with PBS, and continued to culture in complete medium for 2 days. Then Hoechst 33342 (Sigma, St. Louis Missouri, USA) was added at a final concentration of 5 µg/ml and incubate for 5 min. The slides were taken out, mounted with glycerin, and the nucleus morphology was captured with laser confocal microscope (ZEISS, LSM710, Jena, Germany).

Wound-healing assay

Cells (3×10⁵) were seeded in triplicate onto 6-well plates, and cultured for 48 hrs until up to 95% confluence. The cell monolayers were uniformly scratched using sterile pipette tips. The detached cells were removed by washing with PBS, followed by incubation in serum-free medium. At 0 and 24 hrs, the scratched area was photographed under the microscope (ZEISS, Primo VertHD, Jena, Germany).

Cell counting kit-8 (CCK8) assay

The lung cancer cells A549 *vs* A549-D, H1299 *vs* H1299-D, and PC-9 *vs* PC-9-D were collected and counted. 2×10^3 cells were seeded in a 96-well plate and incubated for 48 hrs. 10 μ l/well CCK8 (Dojindo, Kumamoto, Japan) were added to 100 μ l of medium, and incubated for 2 hrs. CCK8 plates were then read at wavelength of 450 nm using a Bio-Tek microplate reader (EON, Vermont, USA).

Colony formation assay

The lung cancer cells A549 *vs* A549-D, H1299 *vs* H1299-D, and PC-9 *vs* PC-9-D were collected and counted. 2×10^3 cells were seeded in a 35mm dish and incubated for 2 weeks. During this period, an inverted microscope was used to observe cells continuously. When cells formed obvious clones under the microscope, the culture medium was removed, and cells were washed with PBS. The fixative (4% paraformaldehyde, Sigma, St. Louis Missouri, USA) was added and incubated for 30 min. After the fixative was removed, crystal violet (Sigma, St. Louis Missouri, USA) was added to stain for 15 min. The clones were counted and their images were photographed.

Statistical analysis

All statistical analyses were carried out using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). The associations between the content of PGCCs and the clinical pathological characteristics of the patients were analyzed by the Chi-squared test. Student's two-sided test was used to compare the significance between control and treated groups. $P < 0.05$ was considered statistically significant in all tests.

Results

Paclitaxel can induce the formation of PGCCs in NSCLCs

We first tested whether paclitaxel can induce the formation of PGCCs in non-small cell lung cancer. Common non-small cell lung cancer cells A549, H1299 and PC-9 (5×10^3) were seeded at an appropriate amount into a 96-well plate. 0, 4.69, 9.38, 18.75, 37.5, 75, 150, 300, and 600 ng/ml of paclitaxel was used to treat these cancer cells for 48 hrs, and the treated cells were then recovered in a regular medium for 48 hrs. CCK8 was applied to detect cell proliferation, and the relative cell viability was calculated (Figure1A). When cells were exposed to PTX (0-600 ng/ml) for 48 hrs and allowed to recover for 48 hrs in regular medium, cell death increased with PTX concentration, and the concentrations that led to 50% inhibition of cell viability (IC50) were 10.5 ng/ml (A549), 38.5 ng/ml (H1299), 1.18 ng/ml (PC-9). We treated cells with the concentrations 75 ng/ml (A549, PC-9), 300 ng/ml (H1299) for subsequent experiments. Further, we observed PGCCs under microscopy. It was shown that only a few PGCCs survived from the treated cells with paclitaxel after 48 h while more than 70% of the treated cells were killed. The surviving PGCCs were then cultured in regular medium to recover for 2 days, and were subjected to stain with Hoechst 33342 and capture the nucleus morphology with laser confocal microscopy. It was revealed that PGCCs became larger (more than three times larger than the control) in morphology, whereas some would continue to die while a part of them would bud and produce daughter cells (Figure1B).

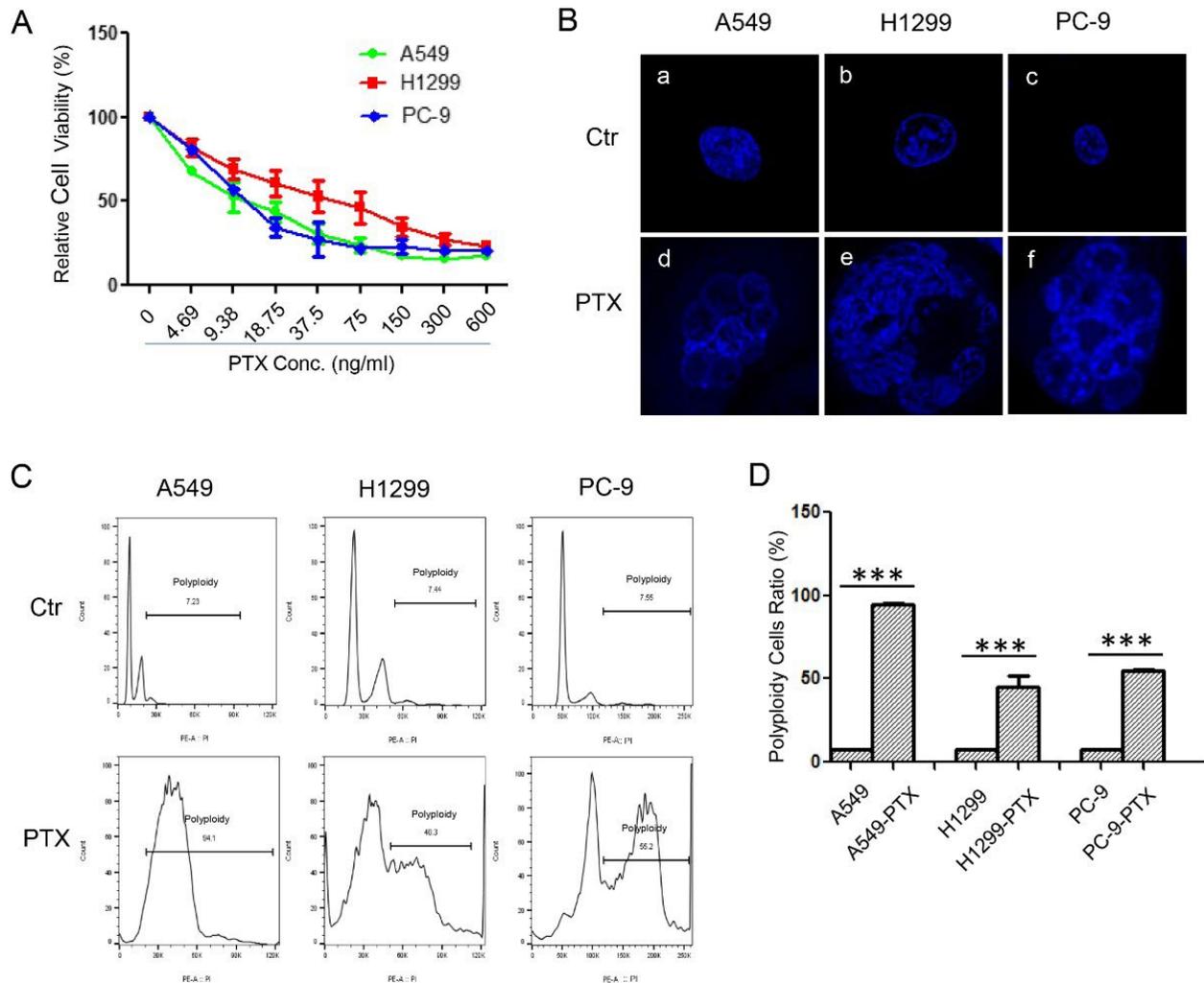


Figure 1: Paclitaxel induces the formation of PGCCs in lung cancer cells. The viability of lung cancer cells upon PTX treatment at varied concentrations was first assessed by CCK8 assay A) The PTX-induced PGCCs were observed under laser confocal microscopy with Hoechst 33342 staining B) comparing to that of controls. Flow cytometry assays further revealed that PGCCs increased significantly in comparison with PTX treatment group and control C) Accordingly, the ratios of PGCCs were statistically shown D) Each bar represents the mean \pm SD of three independent experiments. Student's t-test was used to determine statistical significance: *** P < 0.001.

We further figured out the proportion of PGCCs in the total cancer cells. The recovered cells were collected and subjected to PI staining and flow cytometry assay. The results showed that the contents of PGCCs would increase to 94.1%, 40.3%, and 55.2% in the paclitaxel-treated A549, H1299, and PC-9 cells respectively, comparing to 7.23%, 7.44%, and 7.55% in the untreated controls (Figure1C). Flow cytometry assays further revealed that PGCCs increased significantly in comparison with PTX treatment group and control (Figure1D).

PGCCs bud daughter cells by neosis and PGCCs in NSCLC tissues

The phase contrast microscope was further applied to observe budding of PGCCs. As shown in Figure 2, the morphologic characteristics of lung cancer cells significantly changed before and after PTX treatment.

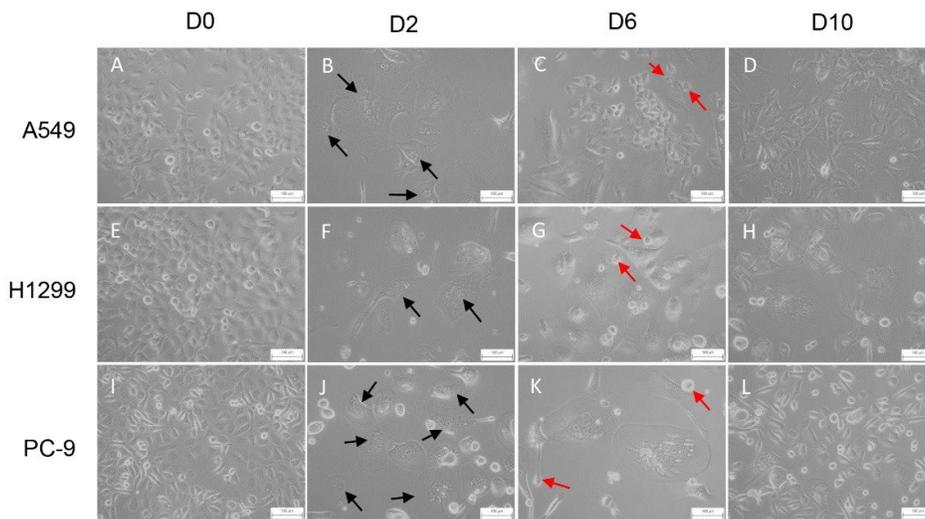


Figure 2: PGCCs with budding daughter cells. The morphologic characteristics of lung cancer cells before and after PTX treatment were observed by phase contrast microscope (200 \times). Comparing to the regular cultured A549 (A), H1299 (E), PC-9 (I) cells, PGCCs (black arrow) were observed to form and survive upon PTX treatment on day 2 (B, F, J), and daughter cells (red arrow) would begin to bud from PGCCs on day 6 (C, G, K) and passage. Many daughter cells were found to surround with large cells at day 10 after paclitaxel treatment (D, H, L).

In this paper, day 0 refers to cells before treatment; day 1 refers to the first day after exposure to PTX (recovery day 1). Comparing the cells without PTX treatment showed a normal growth pattern and were able to divide normally (Figures 2A, 2E, 2I), most of the PTX-treated cells (~70%) were killed in 2 days, and only a small part of PGCCs would survive on the second day of the recovery culture (Figures 2B, 2F, 2J). Of note, PGCCs began to produce small daughter cells on the sixth day of the recovery culture (Figures 2C, 2G, 2K). The daughter cells budding from PGCCs would passage, and a great number of daughter cells were found to surround with large cells on day 10 after recovery culture (Figures 2D, 2H, 2L).

PGCCs in NSCLC tissues

We ulteriorly examined the content of PGCCs in clinical NSCLC specimens and tried to figure out the clinical correlations and significances. 47 clinical non-small cell lung cancer specimens from Clinical BioBank of Shanghai Chest Hospital were enrolled. Tumors were classified according to predominant histologic subtype and grouped by architectural grade (lepidic [LEP], acinar or papillary [ACI/PAP], and micropapillary or solid [MIP/SOL]) based on conventional classification methods [26]. The content of PGCCs and the clinical characteristics relevance was shown in Table 1. PGCCs in these samples were examined under a microscope. Based on the definition of PGCCs established by Zhang et al. [25], cell size measurement and morphological observation showed the presence of PGCCs in lung cancer tissues (Figure 3), which possessed huge or polyploid nuclei.

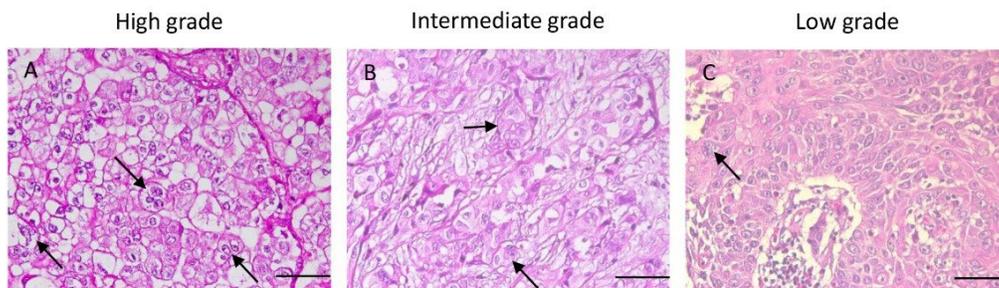


Figure 3: PGCCs in clinical NSCLC specimens. Clinical samples of NSCLC tissue were subjected to perform H&E staining, and PGCCs were examined under a microscope (400 \times). The representative images of PGCCs in high- A), intermediate- B), and low-grade C) of NSCLC were shown (scale bars, 50 μ m).

As shown, the content of PGCCs was related to the diameter of the tumor and the grade of the tumor. The content of PGCCs was significantly higher in tumors with a diameter greater than 3.5 cm than ones less than 3.5 cm ($P = 0.022$; Table 1). Moreover, the content of PGCCs in high-grade NSCLCs was significantly higher than that in low-grade ones ($P = 0.006$; Table 1).

Clinicopathologic	Feature	Number of PGCCs		<i>p</i> value
		Low (< 5)	High (≥ 5)	
All cases	Age	18	29	0.278
	≤ 60	7	16	
	> 60	11	13	
Gender	male	12	15	0.314
	female	6	14	
Diameter of tumor	≤ 3.5	5	17	0.022
	> 3.5	13	12	
Pathological grading	I	4	12	0.171
	II	5	10	
	III	9	7	
	IV	0	0	
Lymphatic metastasis	N0	6	16	0.202
	N1	8	6	
	N2	4	5	
	N3	0	2	
NSCLC typing	low (LEP)	8	2	0.006
	intermediate (ACI/PAP)	8	17	
	high (MIP/SOL)	2	10	

Table 1: The content of PGCCs and the clinical characteristics relevance.

Daughter cells from PGCC budding have a stronger ability of migration, proliferation and colony formation. We further investigated whether tumor cells and their daughter cells have the same biological capability. At first, the lung cancer cells A549, H1299, and PC-9 and the daughter cells of PGCCs A549-D, H1299-D and PC-9-D were inoculated in 96-well plates respectively, and subjected to detect cell proliferation ability at 24, 48, 72, and 96 h by CCK8 assay (Figure 4A). The results showed that the budding daughter cells had stronger proliferation ability than the tumor cells, and significant differences would reach at 96 hrs. Moreover, wound healing assay was performed to detect their migration ability (Figure 4B). It was revealed that significant differences of cell migration ability were appeared only after 24 hrs of post-scratching culture between daughter cells and the tumor cells ($P < 0.05$, Figure 4C). Lastly, we compared the colony formation ability in 35 mm culture dishes. We observed that the daughter cell group formed a larger number of clones that the untreated ones (Figure 4D), and the differences were statistically significant ($P < 0.01$, Figure 4E).

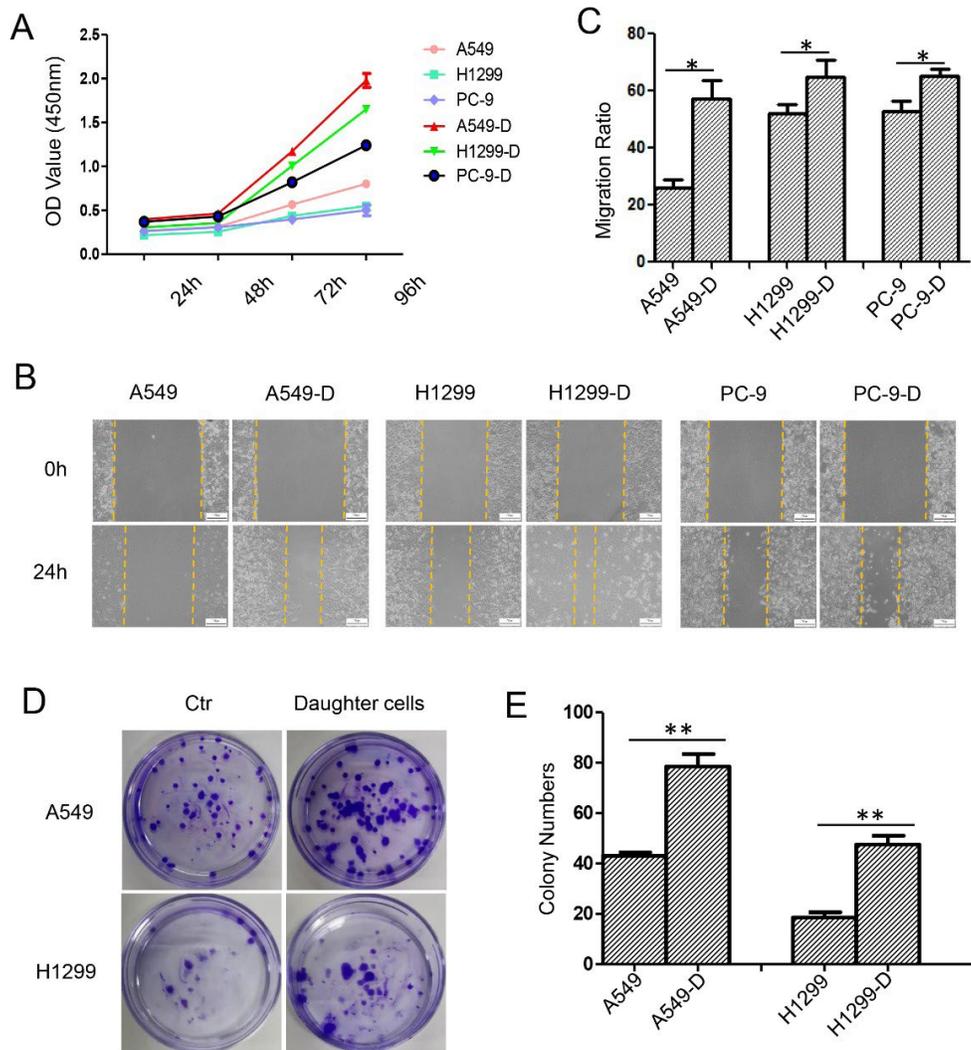


Figure 4: Daughter cells budding from PGCCs have stronger biological capability. The lung cancer cells A549, H1299, and PC-9 and the daughter cells A549-D, H1299- D and PC-9-D were cultured respectively, and subjected to detect and compare their biological capability. CCK8 assay was used to detect cell proliferation ability **A)**, wound healing assay was performed to detect migration ability **B)**, the migration ratio between tumor cells and the daughter cells budding from PGCCs were statistically analyzed **C)** and colony formation assay was applied to assess clone formation ability **D)**. Accordingly, the colony numbers between tumor cells and the daughter cells budding from PGCCs were statistically analyzed **E)**. Student's t-test was used to determine statistical significance: * $P < 0.05$, ** $P < 0.01$.

Discussions

PGCCs could be observed in both tumor cell lines and tumors [27,28]. The expression level of PGCCs was elevated after exposure to tumor therapy [16,29-33]. Studies found that PGCCs would die due to mitotic catastrophe [34-36]. However, increasing evidences showed that PGCCs can undergo neosis to form numerous small mononuclear cells [17,22]. The small mononuclear cells via neosis had a potential role in tumor therapeutic resistance [21,22]. However, the direct role of PGCC-derived neosis in tumor recurrence is poorly understood [20]. Lung cancer is the tumor with the highest mortality rate in the world. The recurrence of lung cancer has always been a difficult point in clinical treatment, and the mechanism of tumor recurrence is still unclear. The reports of PGCCs in the studies are mostly concentrated in colorectal cancer, ovarian cancer, breast cancer, and prostate cancer, but less on lung cancer. Here in above, we focused on PGCCs in non-small cell lung cancer. We found that the content of PGCCs increased significantly after treatment with PTX in NSCLC *in vitro* and *in vivo*.

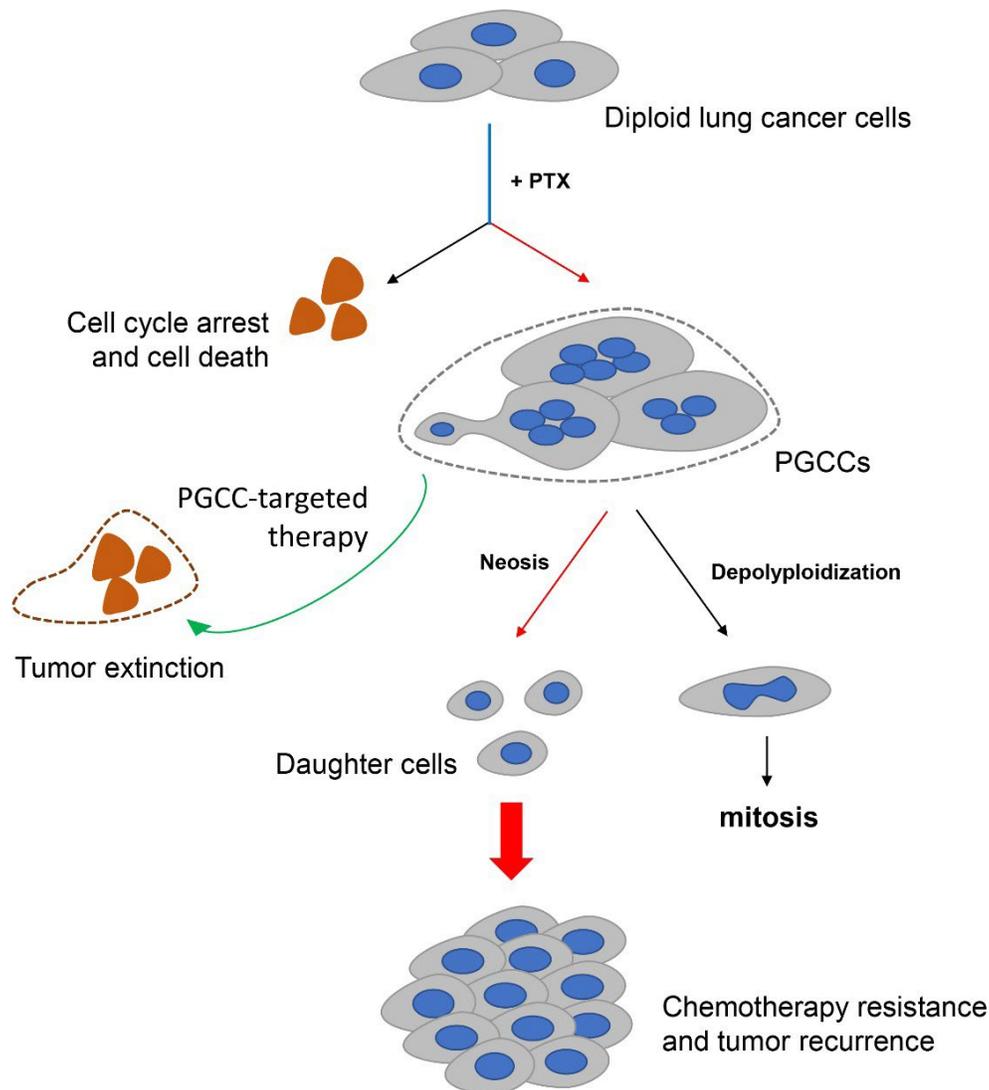


Figure 5: The schematic diagram of PTX-induced PGCCs and its effect on tumor recurrence. When cancer cells are treated with PTX, diploid cancer cells may undergo cell cycle arrest and cell death, and they may enter endoreplication and turn into PGCCs. The PGCCs may survive and bud to form daughter cells, which have stronger biological capability, leading to chemotherapy resistance and tumor recurrence. PGCC-targeted therapy may decrease tumor bulk and open a window for cancer regression.

We analyzed clinical samples using major histological subtypes and structural grades (mural [LEP], acinar or papillary [ACI / PAP] and micropapillary or solid [MIP/ SOL]) [26], and for the first time revealed the polyploid giant cell content among low-, intermediate- and high-grade of non-small cell lung cancer. The results showed that the content of PGCCs in high-grade lung cancer was significantly higher than that in low- grade one ($p = 0.006$). Noteworthy, we found that the content of PGCC was related to the diameter of the tumor ($p = 0.022$), but not the stage of the tumor. PTX works as a first-line chemotherapy agent for advanced NSCLC patients for many years. However, when NSCLC cells treated with PTX, besides undergoing cell cycle arrest and cell death, diploid cancer cells may also enter endoreplication and turn into PGCCs. The PGCCs can survive and bud to form daughter cells. These small daughter cells budding from PGCCs had stronger ability to proliferate and migrate, which in turn caused tumor recurrence. These results indicated that PGCC might be considered as a target for tumor recurrence, and PGCC-targeted therapy might contribute to decreasing tumor bulk, which would open a new window for cancer regression (Figure 5). Undoubtedly, PGCCs are far from clear, more fundamental investigations are highly needed. The advanced technologies, such as single cell multi- omics technologies [37], multicolor flow cytometry [38], next-generation digital histopathology [39], genetic lineage tracing [40,41], and so on, would contribute to a more comprehensive and deeper understanding. Further researches are under way. In brief, paclitaxel can induce the formation of polyploid giant cancer cells in non-small cell lung cancer, which can bud to form daughter cells through asymmetric division (neosis). These small daughter cells had stronger ability to proliferate and migrate, leading to tumor recurrence. Targeting PGCCs or daughter cells would be effective in inhibiting tumor recurrence.

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Author contributions

LT designed the study. PS, HZJ, LLG, and XHY performed the study and participated in the analysis of the data. NZ and KW analyzed the data. HZJ provided the clinical samples and managed their information. LT, and PS co-wrote and revised the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The study was approved by the Ethical Review Board of Shanghai Chest Hospital, and the patient information was kept confidential.

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