Investigation of lung cancer biomarkers through *in vitro* cell-free DNA

Stan Cho

Received March 10, 2024
Accepted July 06, 2024
Electronic access July 15, 2024

Cell-free DNA (cfDNA) is a short piece of DNA that provides comprehensive information about a tumor shed by tumor cells from multiple tumor sites. The application of cfDNA analysis has been shown to have clinical value in detecting molecular proliferation, monitoring disease progression, and predicting treatment response in patients with advanced cancer; thus, cfDNA is well established as a diagnostic and prognostic biomarker in cancer patients. However, the origins and biological significance of cfDNA still need to be investigated. This study aims to investigate the release patterns of cfDNA in response to culture conditions in non-small cell lung cancer (NSCLC) cell lines. To evaluate cfDNA dynamics *in vitro*, we established a method of withdrawing fetal bovine serum (FBS) in cell culture conditions. We found that the concentration of cfDNA was higher during the time of cell duplication. On the other hand, an elongation of the G0/G1 phase during the cell cycle was correlated with an increase in cfDNA levels, suggesting that the G0/G1 phase could be involved in cfDNA release. Moreover, treating lung cancer using chemotherapy agents, such as etoposide and cisplatin, involved DNA damage in cells and resulted in increased cfDNA release. Distinct cfDNA release patterns were also observed during necrosis compared with apoptosis, with larger fragments being released during necrosis. By demonstrating that cfDNA from cancer cell cultures can represent the genetic background of cancer cells, we conclude that understanding the dynamics of cfDNA release can aid in identifying prognostic biomarkers *in vivo*.

Introduction

Circulating DNA fragments released by cells are termed cell-free DNA (cfDNA). More specifically, cfDNA refers to extracellular nucleic acid fragments present in the bodily fluids that are shed from both normal and diseased cells[1]. The size of cfDNA, which can be evaluated by its fragmentation level, indicates their origins and pathophysiological conditions in the body. It should also be noted that cfDNA released into biological fluids contains the same genetic and epigenetic profiles as nuclear and mitochondrial DNA from viable cells, highlighting the possibility of analyzing the genetic profiles of patients by studying their cfDNA[1]. Furthermore, Leng et al. reported that the concentration of cfDNA in patients with non-small cell lung cancer was higher than those found in healthy controls[2].

Cell dynamics are essentially for cancer progression; for instance, Alexander and Cukierman report that cell-matrix adhesion dynamics can be observed to obtain the first signs of cancer. Despite having been discovered more than 80 years ago, the molecular origins and secretion mechanisms of cfDNA remain unclear. Still, several pathways and sources have been reported in literature, including apoptosis, necrosis, pyroptosis, and extracellular vesicles (EVs). Wyllie et al. reported that the DNA extracted from apoptotic cells exhibited similarities to cfDNA. Likewise, necrosis leads to the creation of large fragments of cfDNA ($\sim$10k bp in necrotic cfDNA as opposed to 167 bp in apoptotic cfDNA). Pyroptosis (caspase 1-dependent cell death) leads to excessive cell swelling and subsequent cfDNA release due to the inward flux of water and ions when induced by various stimuli (e.g., caspase activation, immune-inflammatory reaction, or microbial stimulation)[3]. Moreover, EVs have recently been shown to enable the secretion of cfDNA from perfectly healthy cells. For instance, when exosomes are released by fusion between a multivesicular endosome and the plasma membrane, these exosomes release cfDNA found on their surface[1].

Various types of cells release cfDNA into plasma, urine, and other extracellular fluids. In particular, short DNA fragments known as circulating tumor DNA (ctDNA) are released by tumor cells from various tumor areas, and they offer a complete picture of the tumor since ctDNA contains tumor-specific mutations in its genetic information. Recent studies have shown that the concentration of cfDNA in plasma is associated with tumor volume, metabolic activity, metastatic status, and cellular proliferation indices[4]. The importance of cfDNA and its association with tumors also stems from the fact that it is ubiquitous in the bloodstream[5].

The clinical utility of ctDNA analysis has been demonstrated in detecting molecular profiling, disease progression monitoring, and predicting treatment response in patients with advanced-stage cancers. Since cfDNA is DNA secreted from the patients' cells, cfDNA is imbued with the patient's genetic information. Therefore, cfDNA can be expected to be useful as a diagnostic marker, as it is possible to find mutations of oncogenes or tumor suppressor genes by analyzing its genetic information. However, there is still much to be studied about the generation mechanism of cfDNA.

Table 1: The various techniques that can detect ctDNA are listed above, including information about when they are used, on which cancers they are used, and their various limitations[3].

Sr number	Technique Name	Description	Limitations	Cancer Type
1	Quantitative polymerase chain reaction	Amplifies the genes in real-time	Needs standard, prone to errors, primer design depends on results	Non-small cell lung cancer, breast cancer
2	Droplet digital polymerase chain reaction	Water–oil emulsion droplet technology-based PCR	Lower quantification, loss of linearity at a high concentration	Lung adenocarcinomas, squamous cell carcinoma, neck cancer, breast cancer, gastric cancer, and others
3	Beads, emulsion, amplification, and magnetics	Combination of emulsion PCR and flow cytometry ultrasensitive technique	Single mutation per test; lacks standard data	Blood cancer, colorectal cancer
4	Cancer personalized profiling by deep sequencing	NGS-based method for ctDNA detection	For selected alterations across targeted regions	Cervical squamous cancer, bladder cancer, esophageal, lung cancer
5	Whole-genome sequencing	Analysis of the whole genome	Expensive, comparatively low sensitivity and specificity, large amounts of data	Gastric cancer, pancreatic cancer, breast cancer
6	TAm-Sequencing	Identify low-frequency mutations in *ct*DNA	Less comprehensive	Breast cancer, hepatocellular carcinoma
7	Whole exome sequencing	A sequencing-based technique to study protein-coding regions in the genome	Rare variants affect the sensitivity, restricted only to exon regions	Metastatic melanoma, multiple myeloma
8	Whole-genome bisulfite sequencing	NGS-based technique to find out the methylation status of cytosine	It is difficult to differentiate between substitutions and epiallele changes; single reference genomes are not enough to discriminate the changes. It is expensive and generates a large amount of data	Breast cancer, prostate cancer

Previous studies have found that cfDNA exhibits a ladder pattern, ranging from 150 to 1000 base pairs (bp)[6]. Moreover, pertaining to lung cancer, longer cfDNA fragments have been observed in the blood of lung cancer patients compared with healthy individuals.

Studies that have reported elevated levels of DNA sequence mutations in plasma prove the presence of ctDNA. The diagnostics of ctDNA are dependent on size, length, the presence of repeats, and mutations in a normal sequence. The detection of ctDNA is widespread both in technique and in potential application based on cancer type, and further examples are provided above in Table 1.

The analysis of cfDNA can help detect cancer and identify its origin. The application of cfDNA to the detection of cancer is partly made possible by the well-established fact that the concentration of cfDNA in plasma increases proportionally to tumor volume and growth[7]. When quantifying ctDNA as a diagnostic marker in lung cancer, Sozzi et al. reported that the median concentration of ctDNA in patients was found to be eight times higher than in healthy individuals when visualized by real-time quantitative polymerase chain reaction (PCR) amplification of the human telomerase reverse transcriptase gene[8]. However, whether this principle can be applied to cell culture has not been thoroughly investigated. It should be noted that the reproducibility of cfDNA data across different samples and instruments does not exist, and as such, cfDNA is not currently used in clinical practice. The various critical methodological and technical aspects that are related to quantifying cfDNA in the bloodstream are inconsistent[9].

Due to the biological complexity and the presence of many different gene alterations, the potential of cfDNA as a diagnostic and prognostic marker is still under active investigation. In particular, lung cancer is the most lethal cancer worldwide, with a 5-year survival rate of less than 20%. Although lung cancer screening using chest low-dose computed tomography (LDCT) reduces mortality in high-risk patients, LDCT remains underutilized and is associated with risks such as false-positive results and radiation exposure. Therefore, there is an unmet

need for the development of non-invasive approaches to lung cancer[10].

Therefore, based on the principle of cfDNA release and quantification in the bloodstream, we investigated the release dynamics of cfDNA in non-small cell lung cancer (NSCLC) cell lines. We hypothesized that by studying cfDNA amounts and release patterns under various cell culture conditions, including cell cycle synchronization and chemotherapeutic treatment, these findings could be utilized for cfDNA-based analytics in cancer detection and identification of cancer origin[11]. Furthermore, in our study, we propose that the cfDNA dynamics and its biological significance observed *in vitro* have the potential to be applied in clinical settings, potentially serving as a biomarker for prognosis and diagnosis in patients with various types of cancer.

Experimental Setup

Cell lines and Cell culture

NSCLC cell lines, including H3122 and A549, were obtained and grown in the recommended media with 10% Fetal Bovine Serum (FBS- HyClone^{TM}) and 1% penicillin-streptomycin solution (Corning^{TM} Cellgro^{TM}). The H3122 and A549 cell lines were purchased from the Korean cell line bank. Cells were grown at 37°C and 5% CO2 conditions, and all experiments were performed on fresh cell lines with <5 passages.

Cell culture under normal conditions

Cells (105 cells per well) were plated into 6-well plates and cultured for 24 h. Cells were washed twice with sterile phosphate-buffered saline (PBS) and incubated in 3 mL of fresh medium for 4, 8, 12, 16, 24, and 48 h without FBS, respectively.

Procedure

cfDNA Media Collection

Cells were left untouched on a dedicated shelf in the incubator for 7 days prior to medium collection and cfDNA extraction. The culture medium was carefully pipetted off the plate, centrifuged at 4000 RPM x 5 min, and the supernatant was immediately frozen at -80°C, leaving plenty of supernatant to avoid disturbing the cell pellet containing DNA.

cfDNA extraction

The cfDNA from the supernatant was extracted from 200 μL of medium using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. As demonstrated in Figure 1., the process of cfDNA extraction involved (1) collecting media from the cultured cells and (2) centrifugation to remove cell debris. Finally, the remaining media was then treated with proteinase K before we extracted cfDNA. Proteinase K degrades peptide bonds, digests proteins, and decontaminates cell culture samples to prepare nucleic acids; thus, its inclusion in the cfDNA extraction protocol helps to maximize purity.

This method ensured the cfDNA extracted from the cell culture media was not contaminated and enhanced the interpretability of cfDNA dynamics results. The cfDNA concentration was measured using a tape station. The final eluate was collected and stored at -20°C.

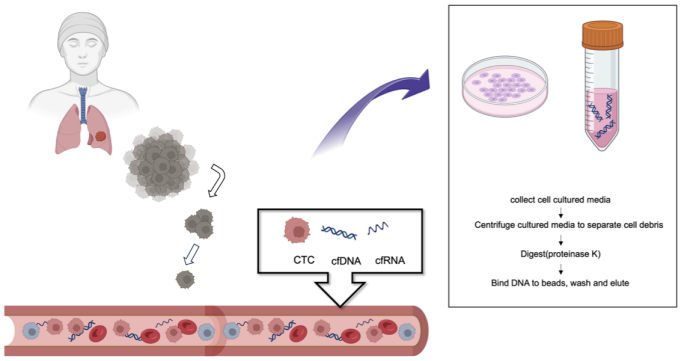

Fig. 1 Schematic diagram of cfDNA media collection

Clonogenic assay

Cells (104 cells per well) were plated into 6-well plates and cultured in a fresh medium. One week later, the surviving colonies were fixed, stained with crystal violet, and counted.

Western Blotting

Whole cell extracts were prepared in lysis buffer, and protein concentration was determined using the BCA protein assay reagent kit (Thermofisher). After 20 μg of total protein was loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), it was transferred to a nitrocellulose membrane. Membranes were incubated with primary antibodies overnight at 4°C with gentle shaking and then incubated with horseradish peroxidase-conjugated secondary antibodies for 2h. Chemiluminescent signals were visualized using Western blotting luminol reagent (Santa Cruz) and exposed to film. Anti-p53, anti-p21, anti-phospho-p53-S15, anti-ac-p53(Lys382), anti-chk1, anti-phospho-chk1(s317), anti-chk2, anti-phospho-chk2(t68) and anti-beta actin.

Polymerase chain reaction

PCR was performed to amplify the EML4-ALK fusion gene. DNA was amplified for 35 cycles at 94°C for 30 s, 60°C for 30

s, and 72°C for 3.5 min, followed by 7 min extension at 72°C.

Measurement of apoptosis and necrosis

Cells were washed twice with PBS and resuspended in 1 × binding buffer at a concentration of 3 × 104 cells/mL. Then, 200 μL of this solution was transferred to a round-bottom tube and mixed with 3 μL of fluorescein isothiocyanate (FITC) Annexin V and 3 μL of propidium iodide (PI) (BD Biosciences). Samples were vortexed and incubated for 15 min at room temperature in the dark. After incubation, cells were analyzed using flow cytometry (BD Biosciences).

Statistical analysis

All experiments in this study were repeated in triplicate, and data are presented as the mean ± SEM. Comparisons between the two groups were made using Student's t-test. Correlation coefficients were calculated using Pearson analysis for continuous variables. In all figures, asterisks denote significance levels as follows: *p $<$ 0.05, **p $<$ 0.01, ***p$<$0.005.

Results and Data Analysis

Dynamics of cfDNA under Various Culture Conditions

While following the cfDNA extraction protocol outlined in previous section., we found that the culture medium can have an impact on cfDNA release. During our study, we suspected that FBS, a commonly used bovine-derived supplement in cell culture, might have influenced the quantity of cfDNA, potentially through the release of cfDNA from FBS itself or its modulatory effect on cell behavior.

We observed that cfDNA exhibited a ladder pattern in both conditions, as shown in Figure 2. (A). However, significantly higher amounts of cfDNA were observed in conditions with FBS compared to those without, as shown in Figure 2. (B). When cultured in the presence of FBS, the cancer cell lines exhibited a greater amount of cfDNA due to both the cfDNA released from tumorigenic cells and the cfDNA obtained from the bovine cells from the FBS. However, in the absence of FBS, the cfDNA is likely to be pure cfDNA derived only from cancer cells. Therefore, subsequent experiments were conducted with FBS withdrawn.

Likewise, we wondered whether the specific cancer cell line derived from patient samples would affect cfDNA measurements. Of note, this finding is crucial when extrapolating *in vitro* findings to clinical contexts where cfDNA is derived from patient samples rather than cell culture media.

Thus, we measured the quantity of cfDNA derived from lung cancer cell lines A549 and H3122 at various time points after subculture (0, 4, 8, 12, 16, 24, and 48 hours). We observed

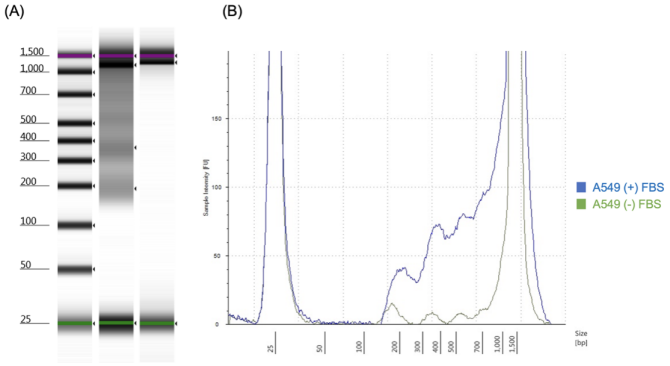

Fig. 2 Quantity and pattern of cfDNA according to FBS with/withdraw conditions. **(A)** Quantity of cfDNA according to FBS with/withdraw conditions. **(B)** Pattern of cfDNA according to FBS with/withdraw conditions.

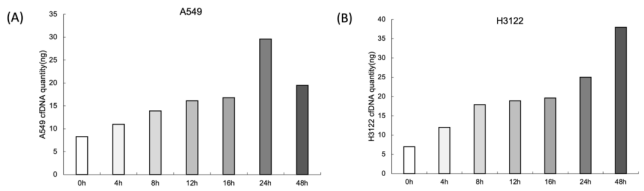

Fig. 3 Cell kinetics of **(A)** A549 and **(B)** H3122 cfDNA release under 0, 4, 8, 12, 16, 24, and 48 hours.

that the concentrations of cfDNA in A549 and H3122 cell lines changed over the time points. For example, we observed in A549 cells that the amount of cfDNA release was highest 24 hours after subculture, as shown in Figure 3. (A). On the other hand, in H3122 cells, cfDNA release was highest 48 hours after subculture, as shown in Figure 3. (B). These findings suggest that cfDNA decayed in A549 cells between 24 and 48 hours after subculture, whereas cfDNA decayed after 48 hours in H3122 cells.

These results also suggest an association between the time of cell doubling and the release of cfDNA in lung cancer cell lines. Considering that the doubling time for A549 cells is approximately 22 hours and that for H3122 cells is about 48 hours, the findings lead to the following interpretation: cells tend to release the most amount of cfDNA at the point of doubling before decay.

Finally, the difference between A549 and H3122 cells in terms of when cfDNA is most released suggests that the release pattern of cfDNA is affected by cell types and their biological processes. Thus, it is crucial to account for cell-specific characteristics when interpreting cfDNA data.

Relationship between cfDNA Release and Phases in Cell Cycle

The mitotic cell cycle is divided into interphase and M phases, allowing for temporal separation of the duplication of cellular content and separation into two genetically identical daughter cells, respectively. Interphase contains the S phase, during which DNA replication occurs. The period of interphase that separates the S phase from the M phase is defined as the "gap phase": the G1 phase occurs before the S phase, whereas the G2 phase occurs after the S phase.

Cells rely on multiple evolutionarily conserved cell cycle control mechanisms to prevent the accumulation and propagation of genetic errors during division. One of such controls is the DNA damage checkpoint. This control occurs throughout interphase, in which cells respond to double-strand DNA breaks via either non-homologous end joining in the G1 phase or homologous recombination in the S and G2 phases.

The decision to exit the cell cycle depends only on the DNA damage checkpoint; in response to irreparable DNA damage, the DNA damage checkpoint can initiate quiescence, senescence, or programmed cell death through transcription factor p53-dependent pathways. It should be noted that p53 mutations are common in cancer, thus preventing cell cycle exiting. Yet, cancer-associated mutations allow for continuous division of cancer cells by compromising their ability to exit the cell cycle [12].

We determined whether the observed differences in cfDNA quantity were due to variations in the cell cycle phases. To this end, we synchronized the cell cycle in A549 cells through serum starvation. Then, we confirmed whether synchronization was successfully achieved by using flow cytometry (FACS) analysis, as shown below in Figure 4. (A). After synchronization, we observed that an increase in cfDNA concentration coincided with an elongation of the G0/G1 phase, as shown below in Figure 4. (B). The G0/G1 phase is the time when metabolic activity is most active in preparation for the S phase, and it can be inferred that the proportional increase in cfDNA abundance during this phase is due to increased metabolic activity [13]. This finding is further corroborated by previous studies; Wang et al. reported that breast cancer cfDNA concentration had a positive correlation to cells that were in the G1 phase. Moreover, much like how several studies have confirmed that cfDNA is released to act as an intracellular messenger, this study found that breast cancer cells had released cfDNA through EVs [14].

This finding shows that the cell cycle and cfDNA dynamics may be potentially associated. The increase in cfDNA concentration during the G0/G1 phase suggests that a particular phase during the cell cycle may alter the rate of cfDNA release. The potential relationship between the cell cycle and the amount of cfDAN release can help identify the underlying mechanisms of cfDNA release in cancer cells. Moreover, the knowledge may further improve the applicability of cfDNA as a biomarker in cancer diagnosis and monitoring.

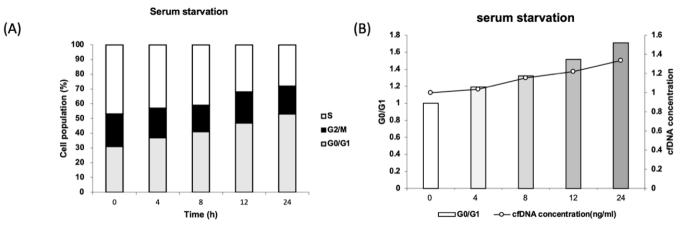

Fig. 4 Correlation between cfDNA quantity and G0/G1 phase. **(A)** cfDNA cell cycle population under 0, 4, 8, 12, 16, 24 and 48 hours. **(B)** cfDNA quantity of G0/G1 phase under 0, 4, 8, 12, 16, 24 and 48 hours.

Still, the underlying molecular pathways need to be explored in further studies. This knowledge may further improve the applicability of cfDNA as a biomarker in cancer diagnosis and monitoring.

Relationship Between Chemotherapeutic Treatment and cfDNA Release

We examined the changes in the quantity and pattern of cfDNA after the cancer cells were treated with etoposide and cisplatin, the two commonly used anti-neoplastic agents for lung cancer chemotherapy.

First, we determined the appropriate drug doses for A549 and H3122 cell lines by using a clonogenic assay. Treatment with etoposide in A549 and H3122 cell lines showed approximately 50% survival rate at 50 μM, as shown in Figure 5. (A). Similarly, when treated with cisplatin, A549 cells exhibited a 50% survival rate at approximately 2.5 μM, while H3122 cells showed the same survival rate at approximately 10 μM, as shown in Figure 5. (B). Thus, we treated A549 cells with 50 μM etoposide and 2.5 μM cisplatin, while we treated H3122 cells with 50 μM etoposide and 10 μM cisplatin.

In p53 wild-type A549 cells, treatment with etoposide, cisplatin, or the combination of the two agents resulted in increased p53 acetylation and phosphorylation and increased p21 expression, which indicates cellular responses to damage, as shown in Figure 5. (C). In contrast, in p53 mutant H3122 cells, increased phosphorylation of Chk1 and Chk2 was observed, which also indicates a cellular response to damage, as shown in Figure 5. (D). The control group refers to p53 wild-type cancer cell lines that are not treated with chemotherapeutic agents. It should be noted that the amount of cfDNA found in control groups is comparatively lower.

Moreover, we found a significant increase in cfDNA levels 24 hours after both cell lines were treated with the anti-neoplastic agents, as shown in Figure 5. (E) and Figure 5. (F), respecitvely. The increased release of cfDNA in A549 and

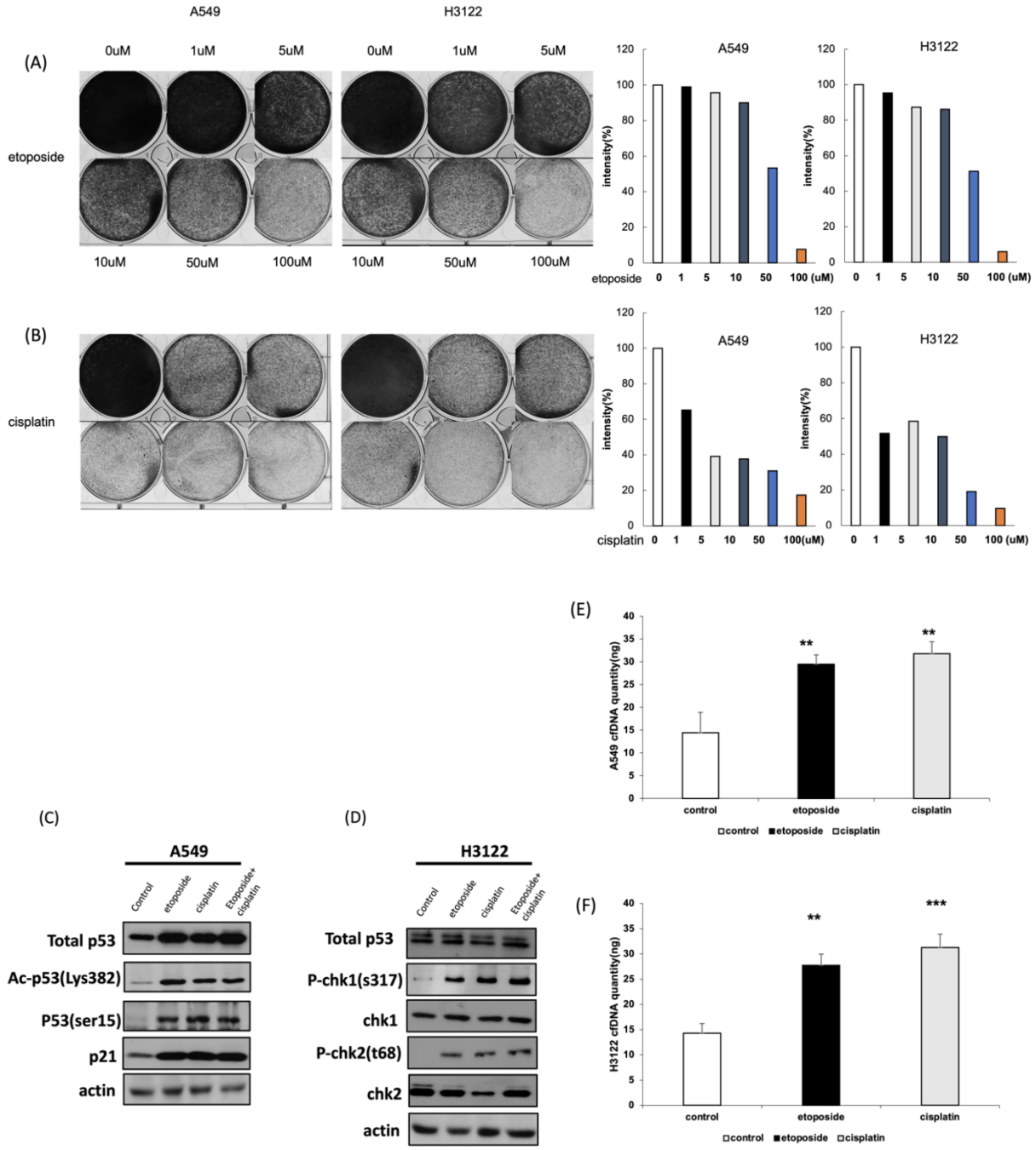

Fig. 5 cfDNA dynamics in relation to etoposide and cisplatin treatment. **(A)** Clonogenic assay shown that etoposide treatment on A549 and H3122 cell line (left) and quantification by image (right). **(B)** clonogenic assay shown that cisplatin treatment on A549 and H3122 cell line and (left) and quantification by image (right). **(C)** Immunoblotting showing protein change in A549 after treatment with etoposide and cisplatin. **(D)** Immunoblotting showing protein change in H3122 after treatment with etoposide and cisplatin. **(E)** A549 cfDNA quantity after treatment with etoposide and cisplatin. **(F)** H3122 cfDNA quantity after treatment with etoposide and cisplatin.

H3122 cells suggests that cancer cells undergoing apoptosis release more cfDNA.

When DNA is damaged by chemotherapeutic agents, DNA undergoes various DNA damage response (DDR) events such

as double-strand breaks, chromosomal fragmentation, translocation, or deletion. During DNA double-strand breaks of chromosomal fragmentation, cfDNA can be derived – since DDR occurs not only in patients but also *in vitro*, it is natural for DNA to be fragmented and released *in vitro*.

In this experiment, we showed that chemotherapeutic agents play a crucial role in modulating the amount of cfDNA released in lung cancer cell lines. This finding also highlights the relationship between the cellular response to damage and cfDNA release. The distinct responses observed in p53 wild-type cells and p53 mutant cells explain that cfDNA release could be associated with apoptosis, emphasizing its potential as a biomarker for monitoring the treatment efficacy of various cancer therapies.

Release of cfDNA via Apoptosis and Necrosis

Previous studies have shown that cfDNA is released as a result of cellular processes such as apoptosis and necrosis. In our study, we investigated the relationship between the amount of cfDNA released and these two distinct forms of cell death.

We created conditions that could induce apoptosis and necrosis. We treated cells with anti-neoplastic agents and allowed for cell overgrowth, which induced apoptotic or necrotic cell death. Subsequently, we extracted cfDNA from cells that had undergone apoptosis or necrosis.

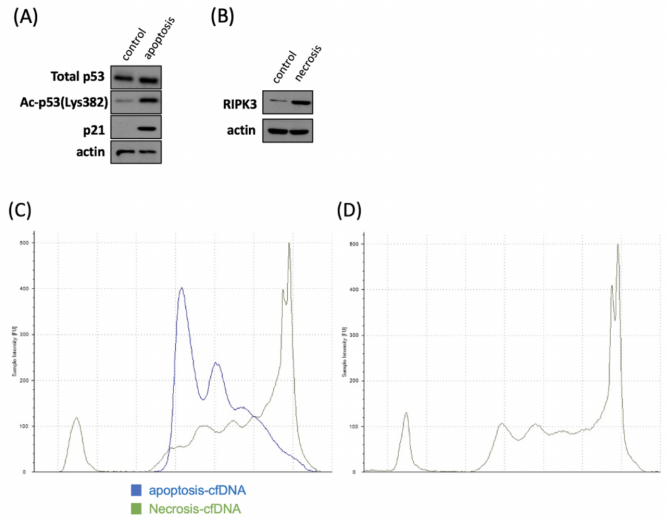

Fig. 6 cfDNA dynamics in relation to apoptosis and necrosis. **(A)** Immunoblotting showing protein change in A549 after inducing apoptosis. **(B)** Immunoblotting showing protein change in A549 after inducing necrosis. **(C)** Sample intensity of cfDNA following apoptosis or necrosis. **(D)** Sample intensity of cfDNA following apoptosis and necrosis simultaneously.

The increase in cfDNA amount in apoptosis was further confirmed by the activation of the apoptotic pathways p53 and p21 pathways, as shown in Figure 6. (A)., which then increases the amount of cfDNA (as DNA is fragmented and released outside the cell). In necrosis, we further confirmed the activation of the necrosis pathway with an increase in RIPK3, a marker associated with cell necrosis, as shown in Figure 6. (B). As previous studies have reported, small-sized cfDNA fragments were predominantly present in cells undergoing apoptosis, forming a distinct ladder pattern. On the other hand, we observed that larger-sized cfDNA fragments were present in larger concentrations in cells that underwent necrosis, as shown in Figure 6. (C).

In apoptosis, mono-nucleosomal fragments predominate, while in necrosis, di- or tri-nucleosomal fragments predominate. This suggests that in apoptosis, there is more fragmentation, resulting in smaller fragments, while in necrosis, there is less fragmentation, resulting in larger fragments. This also raises the possibility that short fragment cfDNA may be detected in patients in the short term following treatment with chemotherapeutics that induce apoptosis. Additionally, in situations where apoptosis and necrosis are induced simultaneously, the proportions of mono- and di-nucleosomes are similar, as shown in Figure 6. (D). This means that simultaneous induction of apoptosis and necrosis synergistically fragments cfDNA.

These findings suggest that cfDNA released through necrosis tends to be larger in size compared to that released during apoptosis. It could be inferred from these findings that the patterns of cfDNA release are different between apoptosis and necrosis. This difference could inform various clinical decisions pertaining to whether cell death occurred predominantly through apoptotic or necrotic processes.

cfDNA Can Reflect the Genetic Background of Cancer Cells

We determined whether cfDNA extracted *in vitro* contained the genetic background of the cells [15]. Moreover, we investigated whether cfDNA could represent the characteristics of cancer cells. To this end, we performed polymerase chain reaction (PCR) analysis on cfDNA.

It is well established that H3122 cells harbor the EML4-ALK fusion, whereas A549 cells do not. Based on this knowledge, we conducted PCR on both H3122 and A549 cells, as well as on cfDNA derived from these cells. After PCR, we found that the EML4-ALK fusion gene was detected only in H3122 cells and their corresponding cfDNA, as shown in Figure 7.

This finding demonstrates that cfDNA could reflect the genetic background of cancer cells and their characteristics. Thus, it is expected that in clinical settings, the specific genetic alterations in cfDNA can be used as non-invasive tools for cancer diagnosis and monitoring.

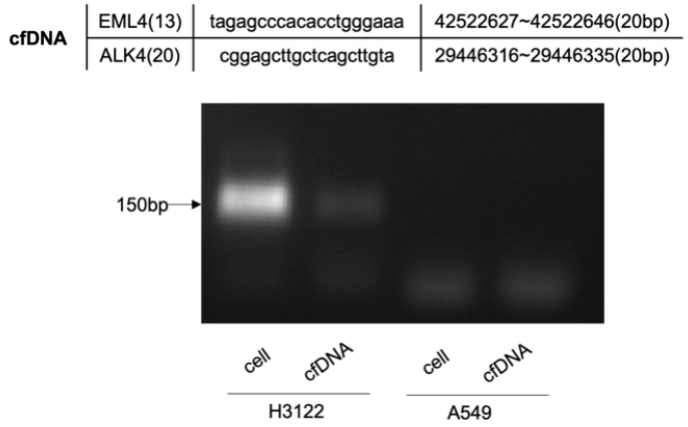

Fig. 7 PCR amplification shows that cfDNA can reflect lung cancer cell line genetic background.

Discussion

It has been understood that cfDNA can have the potential to be used as a biomarker in various diseases. In fact, previous studies have suggested that cfDNA can be used as a tool for noninvasive screening since cfDNA is predominantly found in plasma and serum [16]. Despite the purported applicabilities of cfDNA in various diseases, including NSCLC, evidence was little regarding its pattern of release, except for a few known facts (e.g., cfDNA is released through cellular processes such as apoptosis and necrosis) [17]. Moreover, the current knowledge about the way cfDNA is released is limited predominantly to *in vivo* environments. Thus, in our study, we established conditions for cfDNA extraction from NSCLC in an *in vitro* model. In doing so, we simulated *in vivo* conditions while minimizing the influence of potential confounding factors. We observed a notable increase in the cfDNA level in the presence of fetal bovine serum (FBS) compared with its absence despite similar overall cell numbers. This finding led us to speculate that the increased cfDNA might not originate from cancer cells but rather from fetal bovine DNA.

On the other hand, the differences between A549 and H3122 cell line kinetics could be attributed to the presence (or absence) of the p53 gene. Though we could not perform genetic profiling of the two cell lines due to the limitations of our experimental conditions, we observed that the p53 gene was responsible for the activation of different pathways that respond to cellular damage, thus leading to differences in cfDNA release patterns. However, it is clear that *in vitro* cell lines have limitations, and they are not fully representative of lung cancer subtypes.

Moreover, the mechanism of cfDNA release has remained understudied. Previous studies have found that cfDNA is released more abundantly from apoptotic or necrotic cancer cells than from cells not undergoing the cellular processes [18]. Moreover, it has been known that cancer cell-derived cfDNA tends to have longer DNA fragments. We found that apoptosis, a primary mechanism of cell death induced by chemotherapy, released more cfDNA compared with non-apoptotic cancer cells. More interestingly, we observed that the amount of cfDNA released was highest during the cell's doubling time, which was soon followed by a gradual decay. And yet, the most striking difference was the presence (or absence) of the p53 gene. In Figure 5. (C), A549 and H3122 cell lines each showed activation of different pathways in the presence and absence of p53. This allowed us to infer that the kinetics of the two cell lines differed not only in doubling time but also in their genetic background.

Additionally, an elongation of the G0/G1 phase coincided with an increase in the amount of cfDNA released. Several studies have reported on the ability of cfDNA to diagnose cancer, but little attention has been paid to its release pattern. In this study, we consistently found that the amount of cfDNA increases in the G0/G1 phase in three replicate experiments. The G0/G1 phase correlates to increased metabolic activity that precedes cell division; this study proposed that some pathways activated during the G0/G1 phase released cfDNA. Though the pattern of progressive increase in cfDNA amount in G0/G1 phase or the difference in cfDNA release pattern between different cell lines was confirmed, this study merely serves as a phenomenon report and a deeper exploration of which pathways are activated during the G0/G1 phase and whether the inhibition of these pathways is effective in cancer treatment must be conducted.

Last but not least, previous studies have not thoroughly investigated the differential effect of necrosis and apoptosis on the pattern of cfDNA release [19]. Our study investigated these patterns and found that longer cfDNA fragments are released during necrosis. We suspect that this difference can be attributed to the fact that necrotic cancer cells are less susceptible to degradation by DNase I in blood compared with apoptotic cells since the DNA environment in necrosis is more chaotic and protective against DNase I-mediated degradation. However, the specific mechanism that can explain this observation remains to be studied.

Although cfDNA release from cancer cells exhibits kinetics that change the release pattern and amount of cfDNA depending on various experimental conditions, since the utility of cfDNA as a biomarker lies in how sensitive it is detected and how specific it reflects the genetic background, it can be surmised that cfDNA can be a good biomarker even if the kinetics change.

Still, our study had limitations. First, the *in vitro* model cannot perfectly replicate the clinical condition of real patients. Thus, our results cannot entirely explain the cfDNA release patterns and quantities that will be observed in real patients. Second, components of the tumor microenvironment (including extracellular vesicles, immune cells, or cancer-associated macrophages) can continuously release cfDNA into the extracellular environment, resulting in different concentrations of

cfDNA and, subsequently, tumor-derived DNA. Therefore, it is important to determine the ratio of aberrant to wild-type DNA (including all forms of cfDNA) in order to better assess tumor dynamics, tumor progression, and treatment effectiveness.

Lung cancer screening using chest low-dose computed tomography (LDCT) is still underutilized, and false-positive imaging results and radiation exposure are unavoidable. Therefore, there has been a demand for the development of non-invasive approaches and the development of biomarkers for early detection of lung cancer. This study detected the EML4-ALK mutation via *in vitro* cfDNA as a frequently occurring phenomenon in NSCLC. This suggests the possibility of finding a diagnostic biomarker for noninvasive lung cancer by comparing mutations between normal and cancerous lung cells. It should be noted that *in vitro* cfDNA cannot fully represent biological phenomena observed *in vivo*. To confirm that cfDNA can be detected *in vitro*, the cfDNA obtained from the blood or urine of cancer patients and healthy individuals must be compared via genetic profiling. Still, many studies have reported the use of detecting genetic mutations in patient cfDNA as a possible biomarker, and thus, we recommend further discussion on how small genetic variations can be detected accurately and properly integrated into diagnostic therapies.

Nonetheless, our findings showed that *in vitro* cfDNA has the potential to reflect the genetic background of cancer cells and demonstrate release patterns differently depending on the type of anti-neoplastic agents. Thus, further research is warranted to investigate the release patterns of cfDNA in real patients and explore its potential to be used as a diagnostic and prognostic marker.

References

1 F. S. de Miranda, V. G. Barauna, L. dos Santos, G. Costa, P. F. Vassallo and L. C. G. Campos, *International Journal of Molecular Sciences*, 2021, **22**, 9110–9111, 9116.

2 S. Leng, J. Zheng, Y. Jin, H. Zhang, Y. Zhu, J. Wu, Y. Xu and P. Zhang, *Clinica Chimica Acta*, 2018, **477**, 164–165.

3 S. Hassan, A. Shehzad, S. A. Khan, W. Miran, S. Khan and Y.-S. Lee, *Biomedicines*, 2022, **10**, 2049, 2054.

4 M. Heidary, M. Auer, P. Ulz, E. Heitzer, E. Petru, C. Gasch, S. Riethdorf *et al.*, *Breast Cancer Research: BCR*, 2014, **16**, 421.

5 S.-J. Dawson, D. W. Y. Tsui, M. Murtaza, H. Biggs, O. M. Rueda, S.-F. Chin, M. J. Dunning *et al.*, *The New England Journal of Medicine*, 2013, **368**, 1199–1209.

6 P. Jiang, C. W. M. Chan, K. C. A. Chan, S. H. Cheng, J. Wong, V. W.-S. Wong, G. L. H. Wong *et al.*, *Proceedings of the National Academy of Sciences of the United States of America*, 2015, **112**, E1317–1325.

7 E. Y. Rykova, E. S. Morozkin, A. A. Ponomaryova, E. M. Loseva, I. A. Zaporozhchenko, N. V. Cherdyntseva, V. V. Vlassov and P. P. Laktionov, *Expert Opinion on Biological Therapy*, 2012, **12**, S141–153.

8 G. Sozzi, D. Conte, M. Leon, R. Cirincione, L. Roz, C. Ratcliffe, E. Roz *et al.*, *Journal of Clinical Oncology*, 2003, **21**, 3903–3904.

9 J.-L. Park, H. J. Kim, B. Y. Choi, H.-C. Lee, H.-R. Jang, K. S. Song, S.-M. Noh, S.-Y. Kim, D. S. Han and Y. S. Kim, *Oncology Letters*, 2012, **3**, 925.

10 D. Mathios, J. S. Johansen, S. Cristiano, J. E. Medina, J. Phallen, K. R. Larsen, D. C. Bruhm *et al.*, *Nature Communications*, 2021, **12**, 5060.

11 D. Madhavan, M. Wallwiener, K. Bents, M. Zucknick, J. Nees, S. Schott, K. Cuk *et al.*, *Breast Cancer Research and Treatment*, 2014, **146**, 163–174.

12 H. K. Matthews, C. Bertoli and R. A. M. de Bruin, *Nature Reviews Molecular Cell Biology*, 2022, **23**, 74–88.

13 P. Icard, L. Fournel, Z. Wu, M. Alifano and H. Lincet, *Trends in Biochemical Sciences*, 2019, **44**, 490–501.

14 W. Wang, P. Kong, G. Ma, L. Li, J. Zhu, T. Xia, H. Xie, W. Zhou and S. Wang, *Oncotarget*, 2017, **8**, 43180–43191.

15 B. Silver, K. Gerrish and E. Tokar, *eLife*, 2023, **12**, e83532.

16 S. Gravina, J. M. Sedivy and J. Vijg, *Aging Cell*, 2016, **15**, 398–399.

17 S. Volik, M. Alcaide, R. D. Morin and C. Collins, *Molecular Cancer Research: MCR*, 2016, **14**, 898–908.

18 J. Tuomela, J. Sandholm, M. Kaakinen, A. Patel, J. H. Kauppila, J. Ilvesaro, D. Chen, K. W. Harris, D. Graves and K. S. Selander, *Breast Cancer Research and Treatment*, 2013, **142**, 477–487.

19 S. Grabuschnig, A. J. Bronkhorst, S. Holdenrieder, I. R. Rodriguez, K. P. Schliep, D. Schwendenwein, V. Ungerer and C. W. Sensen, *International Journal of Molecular Sciences*, 2020, **21**, 8062.