Establishment of In-Vitro Cell Cultures Using Patient-Derived Ascitic or Pleural Fluid from Cancer Patients.

***Abstract:***

**Introduction:** Cell culture refers to laboratory techniques that enable the *in-vitro* growth of any kind of cells under physiological conditions. It has tremendous usage in biomedical and clinical research. Ascitic fluid and pleural fluid from cancer patients are excellent sources for cell culture to study metastatic cancers, tumor microenvironments, growth patterns, drug resistance, radiosensitivity, etc.

**Purpose:** Primary aim of the study was to successfully establish *in-vitro* cell cultures using patient-derived ascitic or pleural fluid from cancer patients of Chittaranjan National Cancer Institute, India. Main objective of the study was to check and describe initial cell count (seeding density), average adherence time, morphology of cells, proliferation rate, etc.

**Methods:** Ascitic or pleural fluid samples were collected from 20 cancer patients. After following proper methodologies, 12 successful cultures were taken into consideration. Proper storage and data collection were done simultaneously. Cell block review or fluid cytology reports were also taken to analyze the results properly.

**Results:** In 12 different cultures, only adherence time were quite similar. The seeding densities, proliferation rates, doubling times, etc., were different for different patient samples. Malignancies, N:C ratios, and metastatic conditions were totally different for different cultures. In all cases, results of cultures were similar to the original patient.

**Conclusion:** Our primary and initial data motivate and support us to carry out further research on cell culture and cancer biology to identify drug action mechanisms, drug resistance, radiosensitivity evaluation, etc.

**Keywords:** Cell culture, Ascitic fluid, Cancer, Pleural fluid.

**Introduction:**

Cell culture refers to laboratory techniques that facilitate the in-vitro growth of any kind of cells under physiological conditions. It has exceptional usage in biomedical and clinical research. It was introduced, in the early 20th century, to study the growth and maturation of tissue, vaccine development, drug resistance, production of biopharmaceuticals, etc. In clinical research, cell culture is primarily associated with the development of model systems to explore fundamentals of molecular cell biology, simulating disease mechanisms, or assessing the toxicity of new drug compounds [1]. However, if not properly conducted, cell culture experiments are prone to errors; that’s why good cell culture practice (GCCP) is necessary to assure reproducibility [2]. Cell lines could be classified into three major groups; finite cells, continuous cell lines, and stem cell lines. Finite cell lines have slow growth rates and are derived from the primary cell cultures. Continuous cell lines, also known as indefinite or immortalized cell lines, are generally obtained from cancer cells and achieve high cell densities [2,3]. A cell type may either proliferate in an adherent manner, or it can grow in a free-floating state within a suspension.

Malignant ascites and pleural effusion result from metastatic tumors located in the peritoneal and pleural cavities. Ascitic fluid and pleural fluid from cancer patients are valuable sources for cell culture to study metastatic cancers, tumor microenvironments, growth patterns, drug resistance, etc. [4]. Peritoneal metastasis is typically observed in the case of ovarian, gastric, and pancreatic cancers; whereas pleural metastasis is generally associated with breast and lung cancers. The pathophysiological mechanisms underlying fluid accumulation in these metastatic sites are intricate and involve multiple factors. These fluids from cancer patients contain tumor cells, immune cells, and cytokines useful for primary cultures [5,6]. Metastases in these areas are linked to poor prognoses due to inadequate responses to standard chemotherapy, ultimately leading to organ dysfunction and mortality. So, there is an urgent need to develop strategies to overcome this clinical challenge; and cell culture is essential as it provides a controlled, reproducible environment to study cancer behavior and treatment responses [7,8].

Most of the tumor cell cultures are cultivated with cells obtained from patient-derived tissue specimens. However, acquiring these tissues may be infeasible for patients with metastases as invasive procedures are necessary. In several cases, tumors may be situated in deep anatomical regions that are tough to access for biopsy. Because of such limitations, the culture of tumor cells has proven to be difficult, in patients with metastatic cancers. On the other hand, free-floating tumor cells could easily be accessed in patients with pleural or peritoneal metastases using minimally invasive needle aspiration techniques [9,10]. Many studies have shown that tumor cell cultures could be cultivated in-vitro from patient-derived ascitic and pleural fluid. However, the success rate of these cultures is questionable as it has not been examined thoroughly [11-13]. Besides that, it needs to be studied whether these fluid-derived cell cultures could replicate the therapeutic outcomes of the patients.

In this study at Chittaranjan National Cancer Institute (CNCI), we demonstrated that in-vitro tumor cell cultures could be established from patient-derived malignant ascitic and pleural fluids using a refined protocol for cell culture. We have checked and described initial cell count (seeding density), average adherence time, average doubling time, morphology of cells, contaminations, proliferation rate, confluency levels, etc.

**Aims & objectives:**

* **Aims:** To successfully establish *in-vitro* cell cultures using patient-derived ascitic or pleural fluid from cancer patients of Chittaranjan National Cancer Institute, India.
* **Objectives:**
* To check and describe initial cell count (seeding density), average adherence time, mean doubling time, morphology of cells, contaminations (if any), proliferation rate, etc.
* To store the successful cultures for further studies, specifically to study drug resistance, radiosensitivity, etc.
* To contribute to existing literatures on that particular topic.

**Study setting:**

* Study was conducted at the cell culture laboratory of Department of Molecular pathology in Chittaranjan National Cancer Institute (CNCI), Kolkata.
* We had taken 12 successful cultures out of 20 fluid samples from patients. Their related reports were also collected from other laboratories of CNCI.
* This observational study was performed over a period of six months.

**Inclusion criteria:**

* Confirmed cancer patients older than 18 years.
* Adequate fluid samples (≥50 mL) containing tumor cells.
* Samples that took average adherence time and proliferated successfully [4].

**Exclusion criteria:**

* Contaminated fluid samples and cultures.
* Seeding density below 0.9\*106.
* Fluid samples without tumor or cancer cells, and when proliferation rate was negligible.

**Methodology:**

**Sample collection:**

Patient samples (Ascitic fluid and Pleural fluid) were collected from the doctors of CNCI in the laboratory of Department of histopathology. Then fluid samples were sent to the cell culture laboratory of Department of molecular pathology for remaining procedure. This part of sample collection has been done under the allowance of the ethical committee of Chittaranjan National Cancer Institute. Necessary consent to use patient samples for clinical research purpose had been taken from patients or their family members. This study began after receiving approval from the ethical committee of CNCI.

**Preparation of culture media:**

* Commercially available Dulbecco's Modified Eagle Medium (DMEM) from Thermo Fisher Scientific was used for supporting the growth of cells.
* Adequate amount of FBS (Fetal Bovine Serum) was added.
* Necessary Pen-Strep was added.

**Primary cell culture from fluids:**

* Collection of fluid.
* Cell counts under fluorescence microscope.
* Centrifugation.
* Addition of sufficient media.
* Incubation at 37ºC for 24 hours.
* Replacing media after 24-48 hours.
* Observing the culture under microscope in every day.

**Subculture:**

* Observing the confluency (75-85%).
* Removal of old media.
* Washing step with PBS.
* Addition of trypsin-EDTA for cell detachment.
* Addition of fresh media.
* Centrifugation, resuspension.
* Incubation.

**Storage:**

Properly cultured cells were stored using freezing media. Commercially available cryovials were used for this purpose. Re-culture from frozen stock could be performed for further studies.

**Data collection:**

Patients’ details were collected either directly from patients or from their family members. All other necessary reports were collected from the official reports that were submitted to Department of molecular pathology, Chittaranjan National Cancer Institute.

**Results:**

All collected fluids were pale-yellow colored. Out of 12 successful cultures, 11 were collected from female patients, and only 1 sample was from a male cancer patient. 3/4th of the fluid samples was Ascitic fluids and rest of the samples were pleural fluids. Fluids were taken from several kinds of cancer patients (ovarian, lung, pancreatic, colon, liver) with at least 0.9\*106 seeding densities. 10 out of 12 patients were aged between 50-70. [Table 1]

**Table 1: Details of 12 fluid samples used for Cell Culture**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Patient | Age(Years) | Sex | Cancer type | Fluid type | Collection date(DD/MM/YY) | Seeding density (\*106) |
| P1 | **53** | **Female** | **Ovarian** | **Ascitic** | **13/11/24** | **1.09** |
| P2 | **55** | **Female** | **Ovarian** | **Pleural** | **30/07/24** | **2.3** |
| P3 | **54** | **Female** | **Pancreatic** | **Ascitic** | **03/06/24** | **1.47** |
| P4 | **50** | **Female** | **Uterine** | **Ascitic** | **20/05/24** | **1.15** |
| P5 | **69** | **Female** | **Ovarian** | **Ascitic** | **21/10/24** | **1.92** |
| P6 | **62** | **Female** | **Colorectal** | **Ascitic** | **10/01/25** | **2.18** |
| P7 | **62** | **Female** | **Colorectal** | **Pleural** | **10/01/25** | **2.49** |
| P8 | **83** | **Female** | **Ovarian** | **Ascitic** | **05/06/24** | **1.96** |
| P9 | **42** | **Female** | **Colon** | **Ascitic** | **16/01/25** | **1.74** |
| P10 | **52** | **Female** | **Lung** | **Pleural** | **13/11/24** | **2.38** |
| P11 | **68** | **Female** | **Colon** | **Ascitic** | **02/01/25** | **0.91** |
| P12 | **66** | **Male** | **Liver** | **Ascitic** | **02/01/25** | **1.19** |



**Figure 1: Representative microscopic images of respective cultures from patient 1 to 12.**

In twelve successful cultures, we have observed different types of seeding densities, proliferation rates, confluency. etc. Mean attachment or adherence time of the cells in culture was less than six hours, as expected. Exact number of the tumor cells in pleural or ascitic fluid is very much difficult to detect [4]. In the majority of cases, the samples showed cellular sections with atypical cells showing features such as high nuclear-to-cytoplasmic ratio, hyperchromatic nuclei, irregular nuclear membranes, prominent nucleoli, and moderate cytoplasm, suggestive of malignancy. In many cases, seeding density and proliferation rates were not in positive correlation. Some cases showed features of squamous or glandular differentiation with keratinization and mucinous cytoplasm. Another common observation was presence of reactive mesothelial cells and lymphocytes. Confirmed malignancies were detected in several cases, including metastatic adenocarcinoma and other tumor types. However, few showed no evidence of malignancy, with proliferation rates. We have discussed these findings in elaborated way in the ‘Discussion’ section.

**Discussion:**

**[Overall grammatical errors, punctuations have been revised totally in this section]**

To discuss & analyze properly we also considered cell block review or fluid cytology of the patients [Table 1 and Figure1].

In the case of the first patient, ascitic fluid was collected. Under the microscope, sections were moderately cellular, showing scattered atypical cells in an acinar/glandular pattern having a high N:C ratio, hyperchromatic nuclei, irregular nuclear membranes, and prominent nucleoli with a moderate amount of cytoplasm. The background showed reactive mesothelial cells admixed with small mature lymphocytes. Morphological features were suggestive of malignancy. Seeding density was quite low, but after culturing cells, the proliferation rate was very fast and confluency was very high (fully confluent within a day).

In the second patient, pleural fluid was collected. Under the microscope, sections showed tissue bits lined by hyperplastic squamous epithelium with ulceration and infiltration of the subepithelial tissue by nests of atypical cells having enlarged vesicular nuclei, prominent nucleoli, and moderate eosinophilic cytoplasm. Morphological features were suggestive of malignancy. Individual cell keratinization and keratin pearl formation are seen. Here, seeding density as well as proliferation rate was high.

Ascitic fluid was collected from the third patient. Under a microscope, sections were moderately cellular, showing a fair number of atypical cells and 3-dimensional clusters as well as a glandular/acinar pattern having a high N:C ratio and a moderate amount of cytoplasm. Impression showed positivity for malignancy. Seeding density was moderate, but proliferation rate was low. The confluency was also not high even after weeks.

Ascitic fluid was taken from the fourth patient. Under a microscope, sections showed acini lined by atypical cells having irregular nuclear contour, prominent nucleoli, and moderate eosinophilic cytoplasm. These cells were arranged in small nests as well. The proteinaceous background contained mixed inflammatory cells. Atypical acini were diagnosed in that case. Seeding density and proliferation rate were low. The confluency was not full even after weeks.

In the case of the fifth patient, ascitic fluid was collected. Under a microscope, sections showed small nests of atypical cells having anisonucleosis, pinpoint nucleoli, and moderately abundant pale eosinophilic to vacuolated cytoplasm. Cellular smears showed cells arranged in small round balls with smooth contours and in papillary configuration in a background of reactive mesothelial cells and mixed inflammatory cells. Seeding density was moderate, proliferation rate was high, and more than 70% confluent in three days.

In the case of the sixth patient, ascitic fluid was taken. Under a microscope, sections were moderately cellular, showing a fair number of atypical cells and 3-dimensional clusters as well as a glandular/acinar pattern having a high N:C ratio and a moderate amount of cytoplasm. Impression showed positivity for malignancy. Seeding density was high, but the proliferation rate was moderate. Full confluency was observed within four days.

Pleural fluid was collected from the seventh patient. The left hemicolectomy specimen was collected also for biopsy. Overall histomorphology was consistent with adenocarcinoma with focal mucinous differentiation involving splenic flexure with a maximum dimension of 2 cm, extending up to peri-muscular fatty tissue. All the resection margins were free of tumor. Lymphovascular invasion (LVI) and perineural invasion (PNI) were identified. Metastatic adenocarcinoma was detected. Seeding density was high and proliferation rate was moderate. 70-80% confluency was observed in four days.

Ascitic fluid was collected from the eighth patient. Sections were moderately cellular, showing fair numbers of atypical cells in three-dimensional clusters as well as in an acinar/glandular pattern having a high N:C ratio, hyperchromatic nuclei, and prominent nucleoli with a moderate amount of cytoplasm. Morphological features were suggestive of malignancy. Seeding density was moderate, but proliferation rate was very low in that case.

In the case of the ninth patient, ascitic fluid was collected. Under a microscope, smears were moderately cellular, showing loose clusters as well as scattered reactive mesothelial cells along with atypical-looking cells in tightly cohesive clusters with a vague acinar/glandular pattern having enlarged hyperchromatic nuclei with vacuolated mucinous cytoplasm. The background showed thick mucoid material. Multiple sections showed strips of glandular epithelium lined by atypical cells having a high N:C ratio, hyperchromatic nuclei, prominent nucleoli, and a moderate amount of eosinophilic cytoplasm. Malignancy and metastasis were detected. Seeding density was moderate, but proliferation rate was very high.

Pleural fluid was collected from the tenth patient. Under the microscope, sections were moderately cellular, showing scattered as well as clusters of atypical cells having a high N:C ratio, hyperchromatic nuclei, and prominent nucleoli with a moderate amount of cytoplasm. The background showed scattered reactive mesothelial cells admixed with small mature lymphocytes. Seeding density as well as proliferation rate was high. Full fluency was observed within two days.

Ascitic fluid was collected from the eleventh patient. Under the microscope, sections were moderately cellular, showing scattered reactive mesothelial cells along with small mature lymphocytes. No evidence of any atypia or malignancy was seen in the sections examined. Seeding density was low, and proliferation rate was very low.

In the case of the twelfth patient, ascitic fluid was collected. In biopsy, sections showed fibrocollagenous tissue infiltrated by tumor tissue arranged in sheets, clusters, nests, and trabeculae. These cells were pleomorphic, having hyperchromatic nuclei with prominent nucleoli and moderate eosinophilic cytoplasm. Mitosis and necrosis were noted. Under a microscope, smears showed mixed inflammatory cells, macrophages, and a few reactive mesothelial cells. No malignant cells seen. Seeding density was low, but proliferation rate was moderate. More than half confluency was observed after a week.

Overall, these findings highlight the diverse growth patterns and malignant potential in fluid samples, collected from cancer patients.

**Conclusion:**

Our primary and initial data motivate and support us to carry out further research on that field to identify drug action mechanisms, drug resistance, radiosensitivity evaluation, etc. These findings would contribute effectively to study tumor biology and approximate treatment efficacy. This would enable more effective, targeted cancer treatments and advancements.

**Disclaimer (Artificial Intelligence):**

Authors hereby declare that no generative AI technologies such as Large Language Models and text-to-image generators have been used during the writing or editing of the manuscript.

**Ethical Approval:**

We had taken necessary ethical permission from the ethical committee of Chittaranjan National Cancer Institute. Necessary consent to use patient samples for clinical research purpose had been taken from patients or their family members. This study began after receiving approval from the ethical committee of CNCI.

**Competing Interest**

All the authors reviewed the manuscript before submission and have no competing interest.

**Declaration:**

The manuscript has not been previously submitted or published anywhere else. The full article is original.

**Competing Interests Disclaimer:**

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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