Research on the culture process and establishment of immortal cell lines from peripheral blood of newly diagnosed acute leukemia patients

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ABSTRACT

Objectives: To establish a complete culture protocol for immortalizing blast cells from newly diagnosed acute leukemia (AL) patients, thereby creating a personalized in vitro model. ***Subjects and Methods:*** Peripheral blood samples were collected from thirty newly diagnosed AL patients. Mononuclear cells (MNCs) were isolated and cultured in various media: RPMI 1640 + 10% fetal bovine serum (FBS) + 1x penicillin/streptomycin (P/S), RPMI + 20% FBS + 1x P/S, RPMI + 5% fetal human serum (FHS) + 1x P/S, and StemMACS™ HSC-CFU complete with Epo + 1x P/S. Cell viability, proliferation were evaluated periodically over three months. ***Results:*** The immortalization culture protocol was successfully established using two media: RPMI + 20% FBS and RPMI + 5% FHS. The immortal cell lines from these two media showed a cell doubling time of 48–72 hours. In the StemMACS matrix gel, all cell lines differentiated, while in the 10% FBS medium, two out of three cell lines died after two weeks of culture. The immortalized cells maintained their typical blast morphology and were able to proliferate stably for over 30 passages. ***Conclusion:*** The study successfully established a culture protocol for 3 immortal cell lines from the 30 blood samples of AL patients. This represents a significant breakthrough in blood cancer research in Vietnam, providing a valuable research model for drug screening and the development of personalized treatment strategies, thereby improving treatment effectiveness and patient prognosis.

Keywords: *Acute leukemia; Blast cells; Cell culture; Immortalized cell line*

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INTRODUCTION

Acute leukemia (AL) is a malignant blood cancer with a very poor prognosis. Although current chemotherapy regimens have achieved significant rates of complete remission, the relapse rate remains very high due to the existence of various cell types with different mutations that are prone to drug resistance [1]. To delve deeper into the pathogenesis of AL, culturing cancer cells directly from a patient's biological sample is an advanced technique that allows for the most accurate study of cancer cell biology [2-4]. This technique is, however, extremely complex and challenging. It requires meticulous refinement from the stages of collection, separation, and isolation of cells to finding a suitable culture medium for the cancer cells to live and grow normally as they would in the human body. These cells

are very sensitive to environmental conditions, are prone to death, and face competition for nutrients from other cells [1,5].

It can be said that successfully culturing an immortal cell lines from a patient's samples are high-level technique, demanding meticulous refinement and in-depth expertise. Currently, research on culturing and establishing immortal cell lines from blood cancer patients is very rare in Vietnam. Arising from this reality, this project was carried out with the aim of building a complete protocol to culture and establish immortal cell lines from the blast cells of AL patients. The success of this project will open a new direction in acute leukemia research in Vietnam, as well as providing opportunities for research on other solid tumor cancers, offering an in vitro research model that is personalized, accurate, and of high scientific value.

MATERIALS AND METHODS

Study Objectives

Peripheral blood samples from 30 newly diagnosed AL patients. Inclusion criteria: Patients are diagnosed with AL for the first time. They agree to participate in the study and sign an informed consent form after being fully explained the purpose and procedure. The percentage of blast cells in their peripheral blood is > 20%. Exclusion criteria: Patients who refuse to participate. Patients who have undergone prior chemotherapy. Location: Bach Mai Hospital, Hai Phong University of Medicine and Pharmacy Hospital, Department of Hematology and Blood Transfusion at Hai Phong University of Medicine and Pharmacy. Time: August 2024 - August 2025.

Equipment and materials

Equipment: Class II biosafety cabinet, CO_2 incubator, microscope, refrigerated centrifuge, automated cell counter, Neubauer hemocytometer, cell culture plates: 6-well plates, 35 mm Petri dishes, 10cm Petri dishes. 30 µm filter (Miltenyi Biotec, Germany) Lot: MB130-041-407.

Materials: HES buffer for cell separation, basic culture medium RPMI 1640 with stable Glutamine (Capricorn, Germany, Lot: CP24-7089). Dulbecco's PBS (1x) w/o Ca & Mg, w/o phenol red for washing (Capricorn, Lot: CP24-7407). StemMACSTM HSC-CFU complete with Epo, human, 100 mL (Miltenyi Biotec-Germany, Lot: 5231105330). Heat inactivated, EU-approved fetal bovine serum (Cytiva/Hyclone-USA, Lot: RJ20230003). Fetal human serum (FHS). Penicillin-Streptomycin antibiotic (P/S) (10,000 U/mL) (Thermo Fisher Scientific, Lot: 15140122). Trypan Blue 0.4% (Thermo Fisher Scientific, Waltham, MA, USA, Lot 15250061) for assessing live/dead cell ratio. 30 mm cell culture plates. Red blood cell lysis solution: Red blood cell lysis buffer 10x (Biolegend, Lot: B457850). Cell storage solution CELLBANKER 2 (ZENOGEN, Lot 240405).

Methods

Study design: An experimental, descriptive, and prospective study.

Cell separation and isolation protocol: A 2 mL peripheral blood sample was collected in an EDTA tube and centrifuged at 600 rpm for 10 minutes to separate it into a red blood cell pellet and plasma. The plasma was centrifuged at 2000 rpm for 5 minutes to separate platelet poor plasma (PPP) and platelet rich plasma (PRP). The PPP was then mixed with the red blood cell pellet. HES buffer was added at a 1:1 ratio, mixed well, vortexed for 30 minutes, and left to

settle for 45 minutes. It was then centrifuged again at 200 rpm for 10 minutes to obtain the maximum number of mononuclear cells (MNCs), which were then mixed with the initially separated PRP. Red blood cells were lysed using RBC Lysis Buffer 10X. The buffer was mixed evenly with the separated MNCs, and the mixture was incubated for 15 minutes, then centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded, and the MNCs were washed with PBS supplemented with 10% FBS. Finally, the MNCs were passed through a 30 µm mesh filter. The collected MNCs were then counted and their viability was assessed by diluting them with Trypan Blue solution at various ratios, applying the mixture to a Neubauer improved hemocytometer, and counting under the 10X objective of a light microscope. The mononuclear cells were transferred to a culture plate at a density of 5 x 10^5 cells in 1 mL of culture medium. Experiments were conducted using various culture media, including: RPMI + 10% FBS + 1X PS, RPMI + 20% FBS + 1X P/S, RPMI + 5% FHS + 1X P/S, and StemMACSTM HSC-CFU matrix gel + 1X P/S. All samples were then cultured in a CO_2 incubator at 37°C and 5% CO_2. The medium was changed periodically, and cells were passaged and photographed every 3 days to maintain proliferation and cell evaluation.

Evaluation of biological characteristics

Cell viability and proliferation were assessed every 3 days using Trypan Blue exclusion. Morphology was evaluated by light microscopy, and doubling time was calculated based on cell growth kinetics [2-4].

Data processing and analysis

Excel and GraphPad Prism 10.0 software were used to analyze data and create figures.

Research ethics

The project was approved by the ethics committee of Hai Phong University of Medicine and Pharmacy under protocol number 14/IRB_HPMU.

RESULTS

General characteristics of study objectives

We cultured samples from 30 patients; however, only three representative samples (001, 002, and 008) are presented in this study.

Table 1. *General characteristics of study subjects*

No	Patient ID	Number of White Blood Cells (×10^9/L)	Number of Blast Cells (×10^9/L)	Percentage of Blast Cells (%)
1	001	298	275	92%
2	002	190	180	94.7%
3	008	145	138	95.2%

Table 1 shows that all AL patients had a high white blood cell count, mainly consisting of blast cells.

Results of cell separation and isolation

Table 2. *Cell characteristics after blast cell separation and isolation*

No	Patient ID	White Blood Cells after Separation	Viability (%)

1	001	4.34 x 10^8 cells	98%
2	002	2.84 x 10^8 cells	98%
3	008	4.45 x 10^8 cells	98%

Results of Cell Separation and Isolation

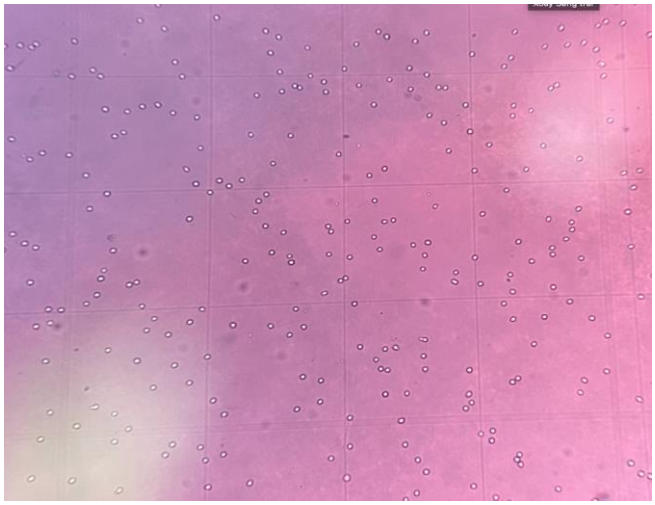
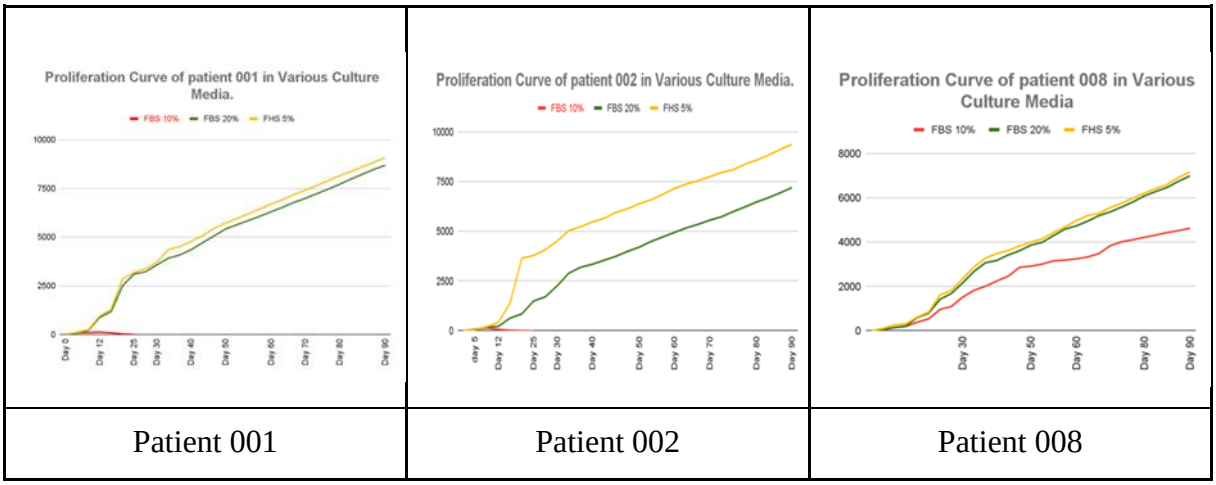


Figure 1. *Image of cells after separation and staining with trypan blue to assess viability*

Figure 1 shows that in the observed microscopic field, 100% of the cells were viable and appeared unstained by trypan blue.

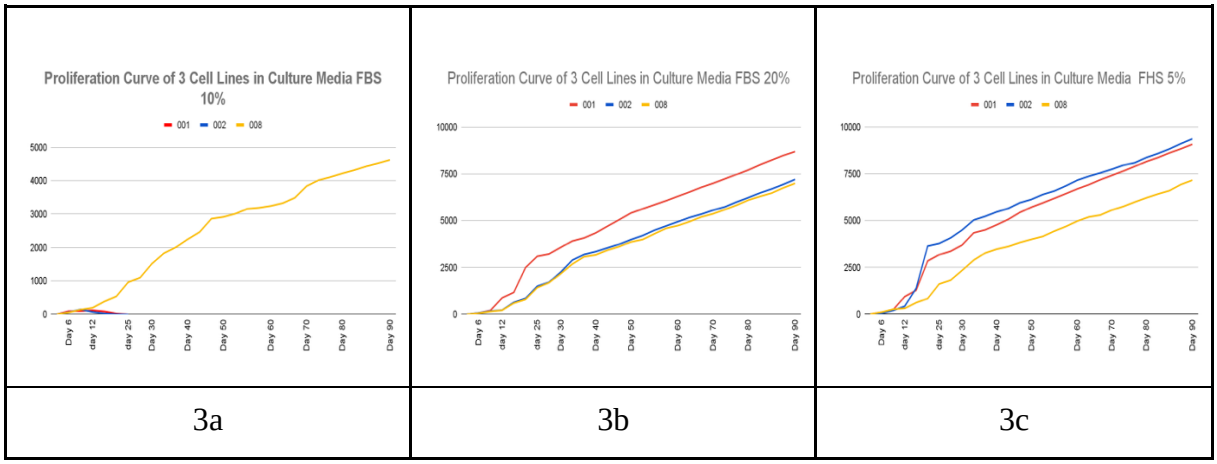
Results of Establishing Immortal Cell Lines



Patient 001	Patient 002	Patient 008

Figure 2. *Proliferation curves of blast cells in vitro from patients 001, 002, and 008 cultured in RPMI 1640 supplemented with 10% FBS, 20% FBS, or 5% FHS (each with 1× P/S).*

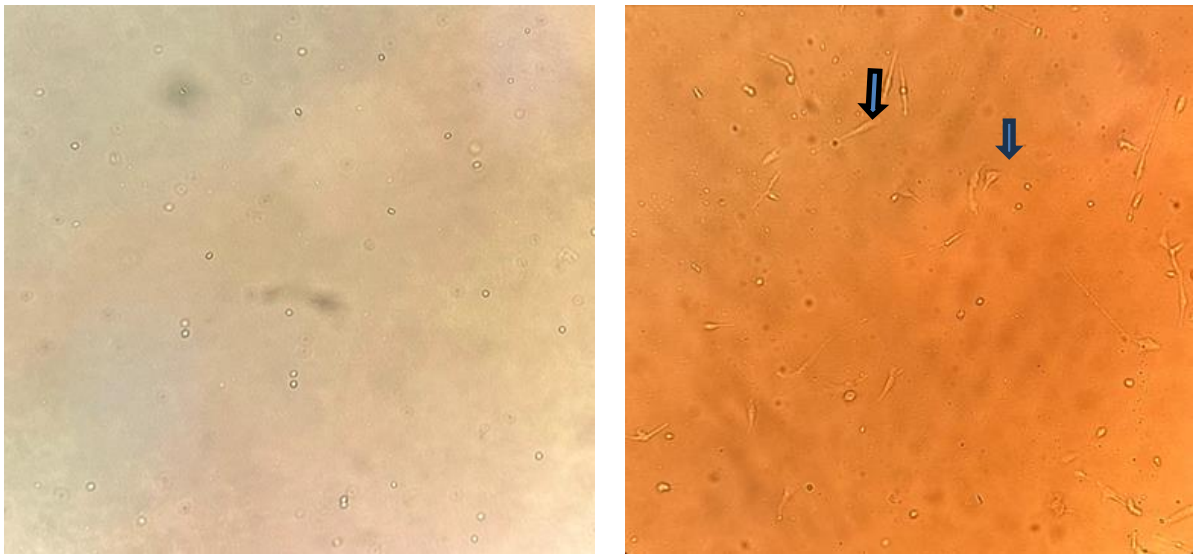
Cells maintained in 20% FBS and 5% FHS media exhibited stable and continuous proliferation, whereas two out of three cultures in 10% FBS medium showed loss of viability after approximately 30 days.



3a	3b	3c

Figure 3. *Proliferation curves of three patient-derived cell lines cultured in different media.*

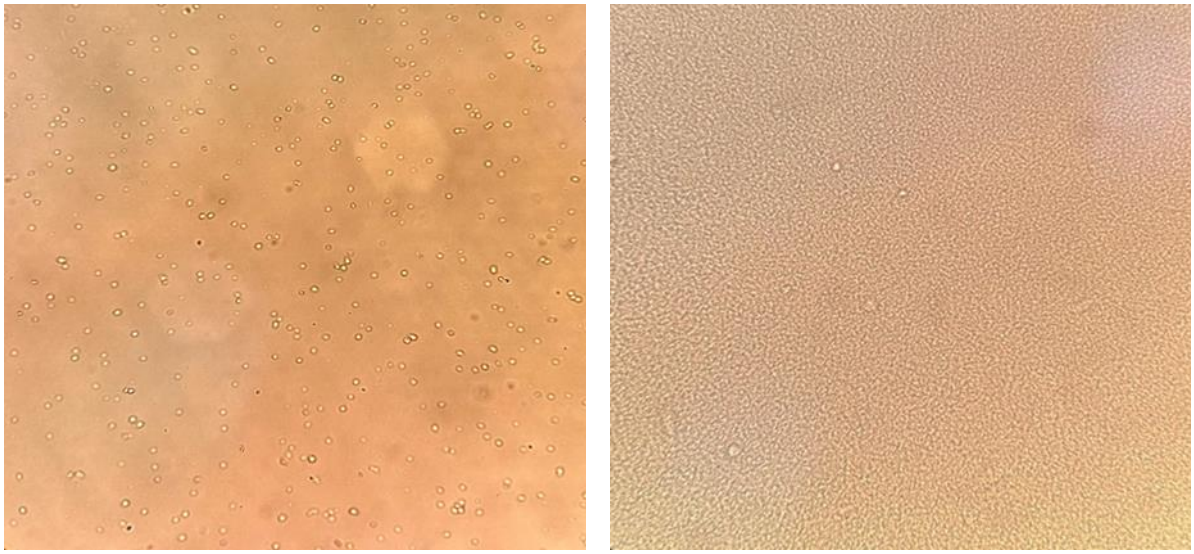
(3a) RPMI 1640 supplemented with 10% FBS and 1× P/S; (3b) RPMI 1640 supplemented with 20% FBS and 1× P/S; and (3c) RPMI 1640 supplemented with 5% FHS and 1× P/S. Cell lines maintained in media containing 20% FBS or 5% FHS exhibited sustained viability and stable proliferation, whereas those cultured in 10% FBS showed reduced survival over time.



Day 1 Day 14

Figure 4. *Image of cell line 002 in StemMACSTM HSC-CFU medium in day 1 and day 14*

The images show the differentiation in blast cell line 002 cultured in StemMACS™ HSC-CFU medium: small, non-adherent cells at day 1 became elongated, macrophage-like, and adherent by day 14. The arrows indicate cells that have differentiated into monocytes and macrophages.



Day 1 Day 21

Figure 5. *The images of cell line 001 in RPMI 1640 + 10% FBS medium in day 1 and day 21*
In day 1, most of cells are aviable, live well, but in the day 21, Most cells had lost viability, with only a few live cells remaining in the microscopic field.

DISCUSSION

Our study aimed to establish a complete culture protocol for immortalizing blast cells from newly diagnosed acute leukemia (AL) patients. While research on this topic has been widely conducted globally, it remains a complex and rarely performed technique in Vietnam.

Characteristics and cell separation protocol: The results from Table 1 and Table 2 indicate that the selected patients all had high peripheral white blood cell counts, with blast cells being the predominant population (over 90%). This is consistent with the clinical features of AL at the time of diagnosis, reflecting a high disease burden. Our cell separation and isolation protocol achieved a high recovery rate, with 98% cell viability. This is consistent with previous studies where the quality of the initial cell sample is considered a crucial factor for the success of the subsequent culture process.

Medium optimization and cell proliferation: The results show that the culture medium is a key factor determining cell survival and proliferation. The RPMI medium supplemented with 10% FBS failed to sustain cell viability. This aligns with international reports stating that a low concentration of FBS is insufficient to support the growth of primary malignant and stem cells. In contrast, RPMI + 20% FBS and RPMI + 5% human serum yielded promising results with stable proliferation, reflecting the supplementary role of growth factors and cytokines from human serum in maintaining the biological characteristics of blast cells. The doubling time of 48–72 hours for these cell lines is comparable to the doubling time of established immortal leukemia cell lines.

The use of StemMAC matrix gel, while not resulting in an immortal cell line, successfully mimicked the bone marrow's extracellular matrix (ECM) and microenvironment, supporting cell viability and differentiation. This highlights the importance of the culture microenvironment and suggests that combining 3D systems could enhance future culture efficiency.

Establishment of immortal cell lines and scientific significance: The key highlight of this study is the successful establishment of an immortal cell line from a patient's AL sample. The stable proliferation over more than 30 passages is a testament to the cell line's immortal nature, paving the way for a personalized in vitro research model. These cell lines can be used for studying disease pathogenesis, drug resistance mechanisms, and preclinical testing, aligning with the approaches of many international research groups.

CONCLUSION

The study successfully established a complete culture protocol and an immortal cell line from the blood samples of AL patients. The RPMI medium supplemented with 20% FBS or 5% human serum proved to be effective, helping the cells maintain stable proliferation and typical morphology. This success is consistent with previous reports on the role of nutrient-rich media and serum in maintaining the viability of malignant blast cells. This represents a significant breakthrough in blood cancer research in Vietnam, providing a valuable in vitro research model for drug screening and the development of personalized treatment strategies, thereby improving treatment effectiveness and patient prognosis.

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