

# ประสิทธิภาพของการใช้เอ็นไซม์ผสมในการผลิตเซลล์เพาะเลี้ยง มะเร็งสมองจากผู้ป่วย

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## Different Enzyme Cocktail Conditions Successfully Establish Primary Brain Tumor Cell Line Derived from Pilomyxoid Astrocytoma Patient

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**หลักการและวัตถุประสงค์:** มะเร็งสมองในเด็กเป็นมะเร็งที่พบมากเป็นอันดับสองรองจากมะเร็งเม็ดเลือดขาว (leukemia) การก่อตัวของเนื้องอกชนิดของมะเร็งสมองในเด็กพบว่า เป็นปัญหาทั่วโลก ส่งผลให้มีพยากรณ์โรคที่แย่มาก การศึกษาเซลล์มะเร็งในระดับโมเลกุลและระดับชีววิทยาเพื่อให้เข้าใจถึงธรรมชาติของเซลล์มะเร็งมีความจำเป็น แต่ปัจจุบันยังไม่มีการสรุปว่าเอ็นไซม์ผสมชนิดใดที่มีประสิทธิภาพมากที่สุดในการผลิตเซลล์เพาะเลี้ยงมะเร็ง การศึกษานี้ทดสอบสมมติฐานที่ว่าเอ็นไซม์ผสมต่างชนิดกันจะมีผลต่อคุณภาพของเซลล์เพาะเลี้ยงแตกต่างกัน

**วิธีการศึกษา:** การเพาะเลี้ยงเซลล์จากเนื้อเยื่อมะเร็งสมองที่ได้จากผู้ป่วยมะเร็งสมองชนิด pilomyxoid astrocytoma โดยใช้เอ็นไซม์ผสมที่แตกต่างกัน 7 ชนิด (C: collagenase, H: hyaluronidase, D: DNase I, C และ D: collagenase และ DNase I, C และ H: collagenase และ hyaluronidase, D และ H: DNase I และ hyaluronidase, และ C และ D และ H: collagenase และ DNase I และ hyaluronidase) สำหรับการแยกเนื้อเยื่อมะเร็งสมองให้เป็นเซลล์เดี่ยวในการศึกษาเปรียบเทียบประสิทธิภาพของเอ็นไซม์ผสมแต่ละชนิดจะศึกษาเปรียบเทียบในแง่ความสามารถในการแยกเซลล์ให้เป็นเซลล์เดี่ยว (degrees of cell dissociation) จำนวนและขนาดของกลุ่มเซลล์ (number and size of clump cells) สัดส่วน

**Background and objective :** Pediatric brain tumor is the second most common cancer after leukemia. Multidrug resistance is a problematic issue worldwide resulting in poor prognosis. Molecular and biological research on cancer cells is essentially required to understand the nature of cancer cells but the most effectiveness of different enzyme cocktail conditions for primary cell line establishment has not been conclusive. This study tests the hypothesis that different enzyme cocktail conditions differently affect the quality of cell line establishment.

**Materials and Methods:** Primary cell line establishment of pilomyxoid astrocytoma tissues was performed using 7 different enzyme cocktail conditions (C: collagenase, H: hyaluronidase, D: DNase I, C+D: collagenase + DNase I, C+H: collagenase + hyaluronidase, D+H: DNase I + hyaluronidase, and C+D+H: collagenase + DNase I + hyaluronidase) for cell dissociation. To compare the effectiveness of 7 enzyme cocktail conditions, a degree of cell dissociation, clump cell size, percentage of live cells, cell proliferation rate and time of 80% cell confluency from each condition were investigated.

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ของเซลล์ที่มีชีวิต (percentage of live cells) ความสามารถในการเจริญเติบโต (rate of cell proliferation) ความสามารถในการเจริญเติบโตให้ได้ร้อยละ 80 (time of 80% cell confluency)

**ผลการศึกษา:** เอ็นไซม์ผสมทั้ง 7 ชนิด มีความสามารถในการแยกเนื้อเยื่อมะเร็งสมองให้เป็นเซลล์เดี่ยวโดยมีเซลล์กลุ่มอยู่บ้างได้อย่างมีประสิทธิภาพเท่าๆ กัน ทุกชนิดของเอ็นไซม์ผสมมีสัดส่วนของเซลล์ที่มีชีวิตในสัดส่วนที่สูงในทุกชนิดของเอ็นไซม์ผสมประมาณร้อยละ 80 ถึง 95 เซลล์ที่แยกโดยเอ็นไซม์ผสมเกือบทุกชนิดสามารถเจริญเติบโตให้มีจำนวน  $1.5 \times 10^5$  เซลล์ได้ภายในเวลา 96 ชั่วโมง หลังจากใส่เซลล์ลงไปเพาะเลี้ยงมีเพียงเอ็นไซม์ผสมชนิด D, H และ C+D ที่ไม่สามารถเจริญจนมีจำนวนที่กำหนดภายในเวลา 120 ชั่วโมง แต่อย่างไรก็ตามเอ็นไซม์ผสมทุกชนิดเซลล์สามารถเจริญเติบโตจนถึงความหนาแน่นร้อยละ 80 ได้ภายใน 120 ชั่วโมง

**สรุป:** ข้อมูลจากการศึกษานี้ชี้ว่า ในการผลิตเซลล์เพาะเลี้ยงมะเร็งสมองจากมะเร็งสมองชนิด pilomyxoid astrocytoma เอ็นไซม์ผสมต่างชนิดกันมีประสิทธิภาพในการผลิตเซลล์เพาะเลี้ยงได้เหมือนกัน

**คำสำคัญ:** การเพาะเลี้ยงเซลล์จากเนื้อเยื่อผู้ป่วย, เซลล์เพาะเลี้ยงเนื้อเยื่อมะเร็งสมอง, การผลิตเซลล์เพาะเลี้ยง

**Results:** All of the 7 enzyme cocktail conditions provided similarly effective cell dissociation. All conditions displayed a high percentage of live cells after dissociation, ranging from 80-95%. Although, most of studied conditions could proliferate to reach  $1.5 \times 10^5$  cells within 96 hours after seeding. D, H and C+D conditions could not proliferate to reach the expected number within 120 hours. However, all conditions effectively grew to produce 80% cell confluency within 120 hours.

**Conclusion:** The present data indicate that in primary cell line establishment of brain pilomyxoid astrocytoma, different standard enzyme cocktail conditions similarly provide the effectiveness of cell line establishment.

**Keywords:** primary cell culture, brain tumor cell line, cell line establishment

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## Introduction

Cancer is the leading cause of death worldwide. Many thousand people are diagnosed cancer everyday. In the USA, 1 in 330 people develops cancer before the age of twenty. Additionally, approximately 13,500 children were diagnosed cancer each year<sup>1</sup>. The aggressive malignant diseases of childhood increase up to almost 30% of all diseases compared to the last 20 years<sup>2</sup>. One in five of children with cancer dies within 5 years after diagnosis. In addition, one-third of cancer children live with long term sequelae such as cognitive impairment, permanent immune suppression and growth defect<sup>3</sup>. Pediatric brain tumor (PBT) is the second most common cancers occurred in children after leukemia. Approximately half of all pediatric cancer patients are diagnosed either leukemia or brain tumor. In addition, brain and central nervous system tumor are the most common solid cancers presented in children<sup>4</sup>. Currently,

medical technology is progressively improved to increase the effectiveness of treatment and decrease side effects. However, no new protocol for treatment of brain tumors has been developed yet during the last 30 years. Conversely, hematologic malignancy such as leukemia has advanced developed therapy; autologous bone marrow transplantation and stem cell transplantation<sup>5</sup>. Besides, the survival rate of pediatric brain tumor patients is still low and treatment outcome is poor<sup>6</sup>. Therefore, molecular knowledge of brain tumor is essentially required for extensive studies to improved treatment outcome and decrease treatment sequelae.

Primary cell culture is exceedingly precious tools for researchers to investigate molecular basic knowledge in many fields especially complex organisms such as brain tumors. Cell culture represents the original tissues from which they are derived. Hence, preclinical *in vitro*

study is effectively investigated in primary cell culture prior to apply to animal models and clinical trials. Basically, a primary cell line is established using 2 methods; mechanical dissociation and enzymatic dissociation. However, mechanical dissociation provided low success rate of establishment approximately 8.05%<sup>7</sup>. Conversely, a high success rate was found in enzymatic dissociation for establishment, approximately 85%<sup>8,9</sup>. Presently, several techniques have been optimized for tissue dissociation in a primary cell line establishment. Therefore, different research centers use different protocols for establishing primary cell lines. The important process of primary cell line establishment is that the more dissociating cells into single cells, the better growing cultured cells. The tissue dissociation using enzymes is commonly used because of better efficiency, viability and less toxicity compared to mechanical dissociation. Further different doses and time of incubation were previously examined to obtain the most appropriate dose and incubation time (Table 1)<sup>10</sup>.

In 1995, Agamanolis and Malone successfully established primary cell lines from 47 PBT specimens. The specimens were enzymatically disaggregated overnight in 200 U/ml of collagenase in tissue culture medium<sup>11</sup>. In 1996, Ali-Osman and his colleagues successfully established glioma brain tumor cell lines using enzymes to dissociate brain tissues, which were DNase type Ia, neutral proteinase and collagenase. They revealed that low grade gliomas and normal brain cells grew slowly in primary cells culture and they required 3-4 weeks to achieve full confluency<sup>7</sup>. However, some researchers can also successfully established primary human cell lines without using enzymes. In 1996 and 2004, Xu and colleagues established 161 new cell lines derived from pediatric brain tumors. Only 5 cell lines were successfully established but only 3 cell lines could grow well in culture media, which were anaplastic medulloblastoma, atypical teratoid rhabdoid tumor and glioblastoma tumor<sup>8</sup>. These lines of evidence indicate that different enzyme cocktail conditions may affect the effectiveness of cell line establishment depending on the tumor types.

To explore the molecular and cellular properties of pilomyxoid astrocytoma, establishment of primary cell

lines is necessary. Although, many standard enzyme cocktail conditions have been used to establish several primary cell lines in Thailand including Khon Kaen University, the most effective one for brain tumor cell line establishment has not been reported. The present study test the hypothesis that different enzyme cocktail conditions differently affect the quality of primary cell line establishment.

## Materials and Methods

### Brain tumor tissues for primary cell line establishment

This study was approved by the Human Ethics Committees, Khon Kaen University (project code: HE 551016). Brain tumor tissues examined in this study were derived from 9-month-old Thai girl patient pathologically diagnosed Pilomyxoid astrocytomas, which is a primary brain tumor. The patient was scheduledly admitted at Srinagarind Hospital for craniotomy with tumor removal. The details of patients are described in the Table 2.

### Seven different enzyme cocktail conditions

The details of 7 different enzyme cocktail conditions are shown in the Table 3. A hundred units of each enzyme were used for cell dissociation.

### Cell dissociation and clump cell size

Brain tumor tissues removed from the patient were cleaned and removed blood clots, blood vessels, necrosis of gross brain tissues and other visible contaminations then they were washed in phosphate buffer saline (PBS). Tumor tissues were finely minced and incubated in 7 different enzyme cocktail conditions; 1) collagenase, 2) hyaluronidase, 3) DNase I, 4) collagenase + DNase I, 5) collagenase + hyaluronidase, 6) hyaluronidase + DNase I, and 7) collagenase + DNase I + hyaluronidase for 2 hours to dissociate cells into single cells. After cell dissociation, they were plated in T25 flasks containing Dulbecco's Modified Eagle Medium (DMEM) with 15% Fetal Bovine Serum (FBS) then 6 different fields were captured from dissociated cells under the light microscope to present the degrees of cell dissociation. The average size of clump cells was also investigated using image J program for measuring clump cell size. Finally, established cell lines were incubated at 37°C in 5% CO<sub>2</sub> and 95% humidity incubator.

**Percentage of live cells**

After cell dissociation process, tumor cells from 7 different conditions were stained with trypan blue to evaluate the number of live cells. Dissociated cells were counted duplicately using hemocytometer to determine number of total cells and live cells then percentage of live cells was analysed.

**Time of cell proliferation**

After cell dissociation,  $2 \times 10^4$  cells from 7 different conditions were plated into each well of 6-well plate for 5 wells and incubated at  $37 \text{ }^\circ\text{C}$  in 5%  $\text{CO}_2$  and 95% humidity incubator for 5 days. Cells were detached and counted number of cells daily to evaluate time of cell proliferation to have  $1.5 \times 10^5$  cells.

**Time of cell growth to 80% confluency**

$2 \times 10^4$  cells from 7 different conditions were duplicately plated in six-well plate then cells were incubated at  $37 \text{ }^\circ\text{C}$  in 5%  $\text{CO}_2$  and 95% humidity incubator. Cells were observed every 24-hour until they reach 80% confluency.

**Statistical analysis**

Statistical analysis was performed using SPSS statistical software version 11.5. All quantitative data were presented as means  $\pm$  standard error of mean. To compare the differences among groups, one-way ANOVA was used. The differences were considered to be statistically significant when p-value is less than 0.05.

**Results****Degrees of cell dissociation**

After brain tumor tissues were incubated in 7 different enzyme cocktail conditions. Degrees of cell dissociation were not different among 7 different conditions. All 7 conditions provided similar degrees of cell dissociation with some clump cells (Figure 1). Single, double or triple enzyme cocktail conditions showed similarly effective disaggregation of brain tumor tissues into single cells.

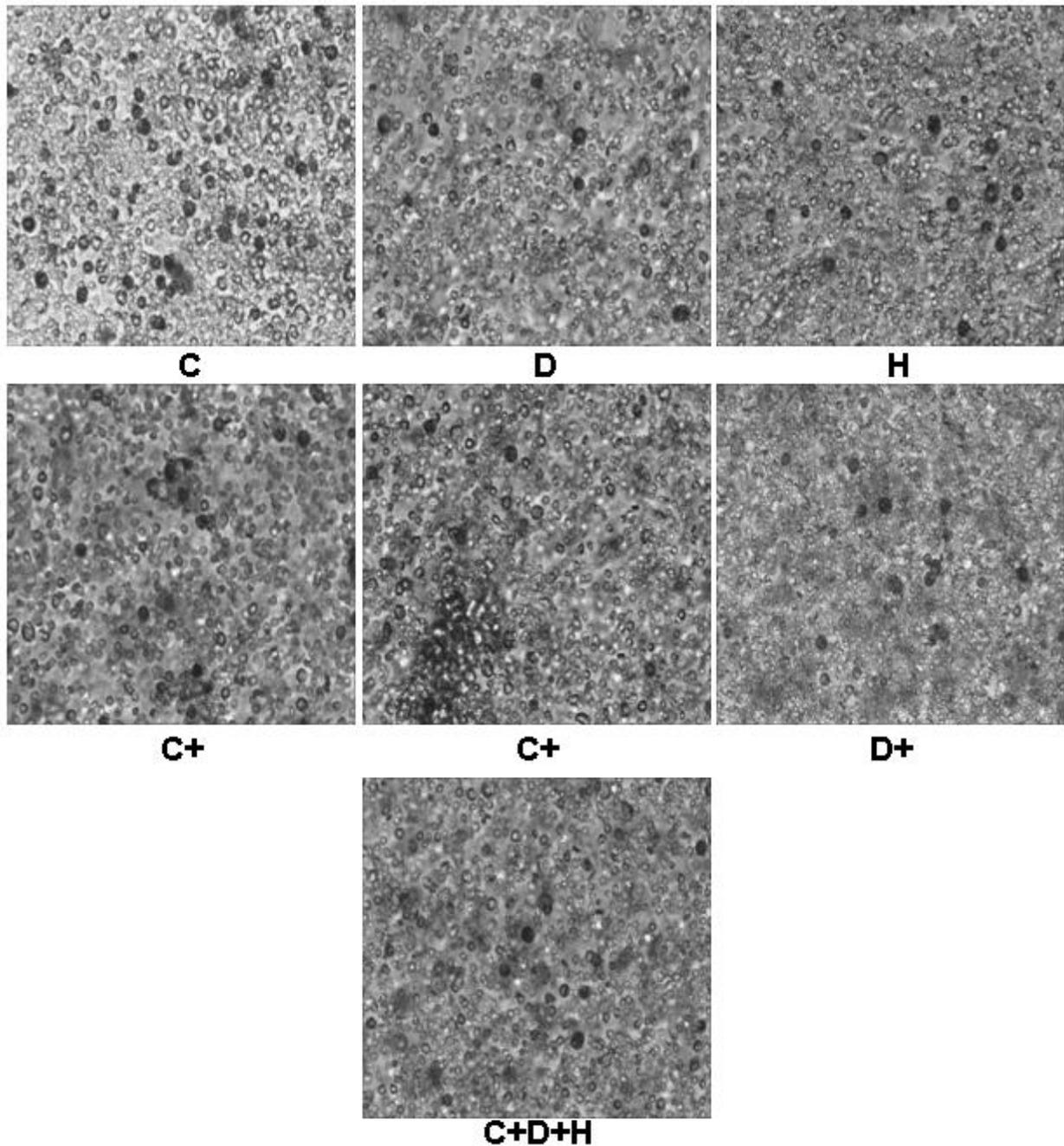
**Clump cell size and number of clump cells**

Clump cells were averaged sizes from captured images of dissociated cells after incubating in different 7 conditions (Figure 2). All conditions provided vary sizes of clump cells. Sizes of clump cells were  $5,435.51 \pm 5188 \text{ } \mu\text{m}^2$  for C condition,  $4,045.02 \pm 2,861 \text{ } \mu\text{m}^2$  for D condition,  $6,694.14 \pm 3,312 \text{ } \mu\text{m}^2$  for H condition,  $4,273.91 \pm 3323 \text{ } \mu\text{m}^2$  for C + D condition,  $10,738.75 \pm 6,255 \text{ } \mu\text{m}^2$  for C+H condition,  $4,578.49 \pm 4,457 \text{ } \mu\text{m}^2$  for D+H condition, and  $1,790.88 \pm 1,718 \text{ } \mu\text{m}^2$  for C+D+H condition (Figure 3). The average size of clump cells from all conditions was approximately  $5,000 \text{ } \mu\text{m}^2$ . C+H condition showed the biggest average size of clump cells, whereas C+D+H condition provided the smallest average size of clump cells compared to other conditions. However, the average size of clump cells from all conditions was not significantly different and clump cells from all conditions had a huge variation in size resulting in large SD. Additionally, the highest number of clump cells was found in C+H condition.

Dissociated cells were also examined number of clump cells. Clump cells, which are larger than  $300 \text{ } \mu\text{m}^2$ , were counted (Figure 2). The number of clump cells was approximately 20-22 clumps in 10 fields of  $5 \times$  magnification. The highest number of clump cells was found in D+H condition (40 clumps) whereas C+D and C+H condition provided the lowest number of clump cells (20 clumps).

**Percentage of live cells**

The percentages of live cells indicate the toxic effects of enzymes after cell dissociation with different conditions. The percentages of live cells from 7 different enzyme cocktail conditions were  $79.22 \pm 2.53\%$  for C condition,  $82.13 \pm 11.62\%$  for D condition,  $95.56 \pm 0.42\%$  for H condition,  $84.09 \pm 9.64\%$  for C+D condition,  $90.82 \pm 3.62\%$  for C+H condition,  $93.59 \pm 1.35\%$  for D+H and  $95.45 \pm 3.07\%$  for C+D+H condition (Figure 3). The percentages of live cells from 7 conditions were not significantly different ( $p = 0.12$ ). Their percentages were ranging from approximately 80-95%. Cells dissociated with C+D+H still have a high percentage of live cells (95%) compared to single or double enzymes used.

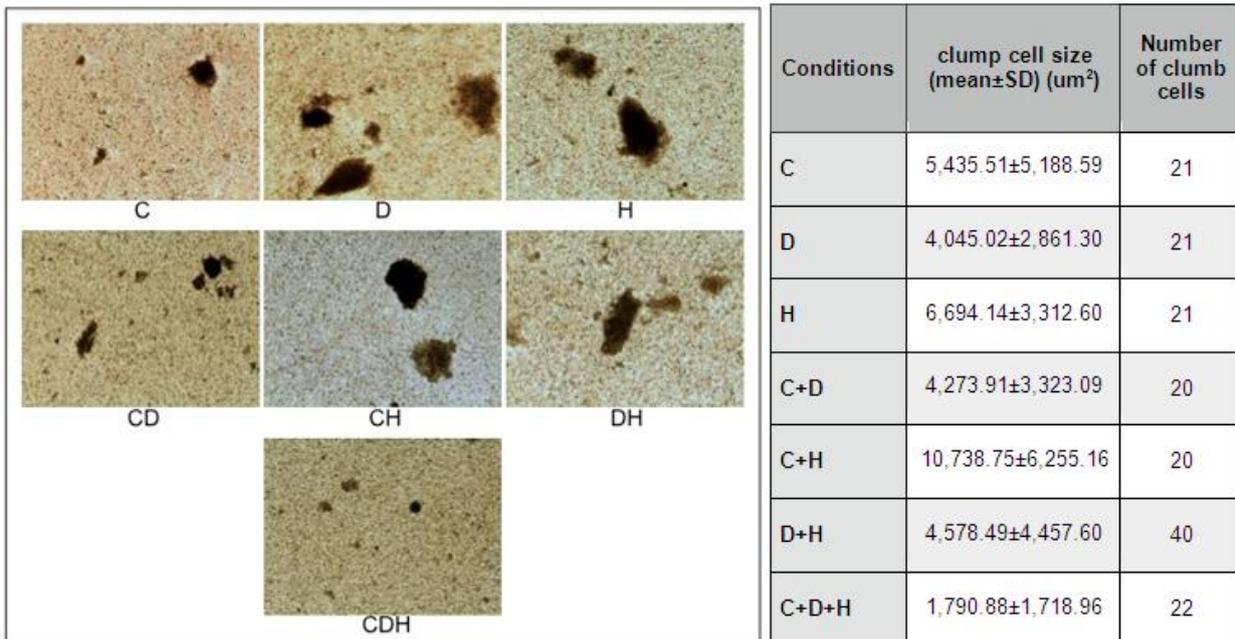


**Figure 1 Degrees of cell dissociation.** Brain tumor tissues were incubated in 7 different enzyme cocktail conditions (C; collagenase, D; DNase I and H; hyaluronidase). All conditions provided effective cell dissociation of brain tumor tissues into single cells with some clump cells (x32 magnification).

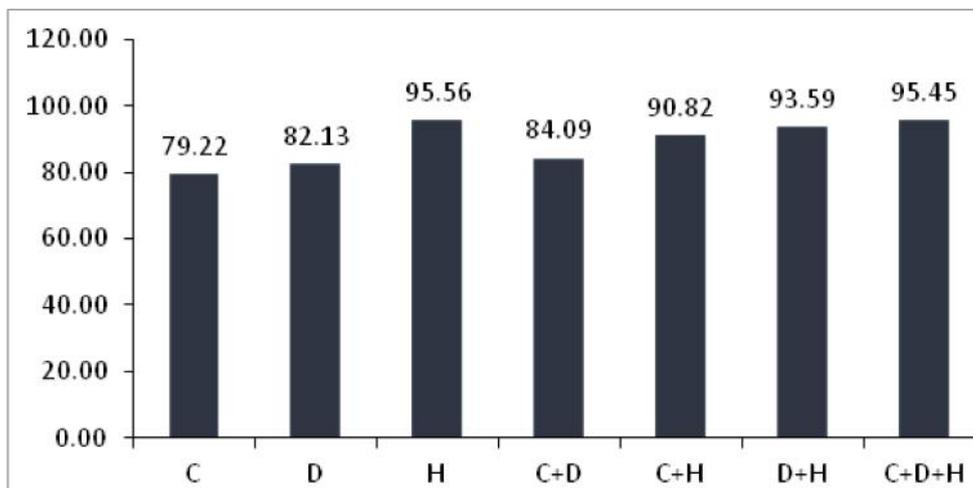
The highest percentage of live cells (95.56%) was detected in H condition whilst C condition provided the lowest percentage of live cells (79.22%) (Figure 3).

**Time of cell proliferation**

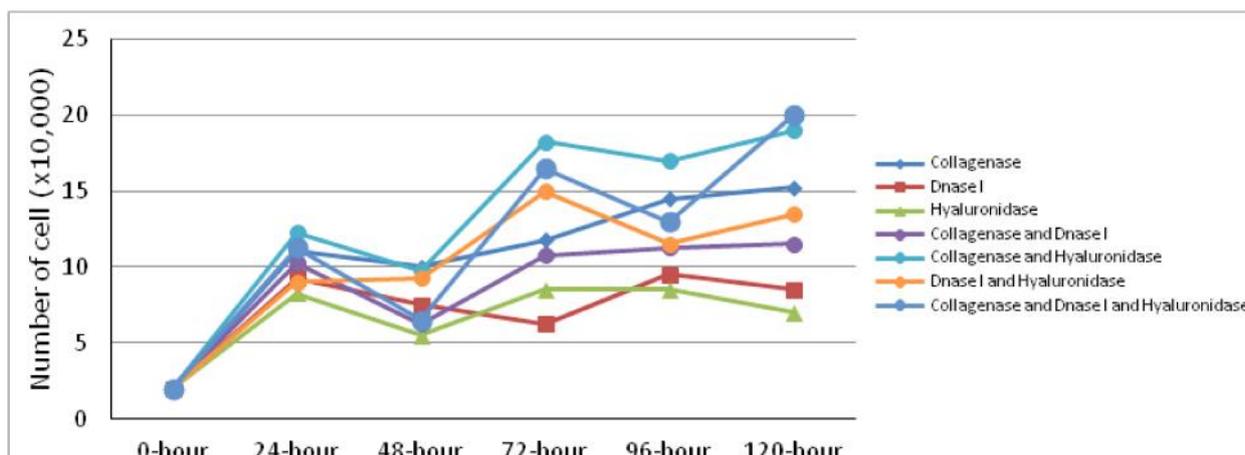
C+H, D+H and C+D+H conditions required 72 hours for growing to the expected number while C condition took 96 hours to reach such number. However, three



**Figure 2 The representative clump cells from all conditions and the average size and number of clump cells.** All conditions provided dissociated cells with different size of clump cells (C; collagenase, H; hyaluronidase and D; DNase I) shown in the figure with 5x magnification. The table shows the size and the number of clump cells from all conditions. Clump cell number from all conditions is presented in the table. Clump cells were counted approximately 20-22 clumps/PF from all conditions and D+H condition had the highest number of clump cells.



**Figure 3 The percentages of live cells from 7 different enzyme cocktail conditions.** The graph shows percentages of live cells after cell dissociation with different conditions (C; collagenase, H; hyaluronidase and D; DNase I). All conditions provided high percentage of live cells ranging from 80% to 95%. C condition had the lowest percentage of live cells (79.22%) whereas both H and C+D+H conditions similarly provided the highest percentage of live cells (95%).



**Figure 4 Proliferative rate of cells incubated in different enzyme cocktail conditions.** Only three conditions; D, H and C+D conditions could not proliferate to reach  $1.5 \times 10^5$  within 120 hours after seeding. C+H, C+H and C+D+H conditions required 72 hours to reach the expected number while C condition needed slightly longer time (96 hours).

conditions; D, H and C+D conditions, could not reach  $1.5 \times 10^5$  cells within 120 hours (Figure 4).

#### Time of cell growing to 80% confluency

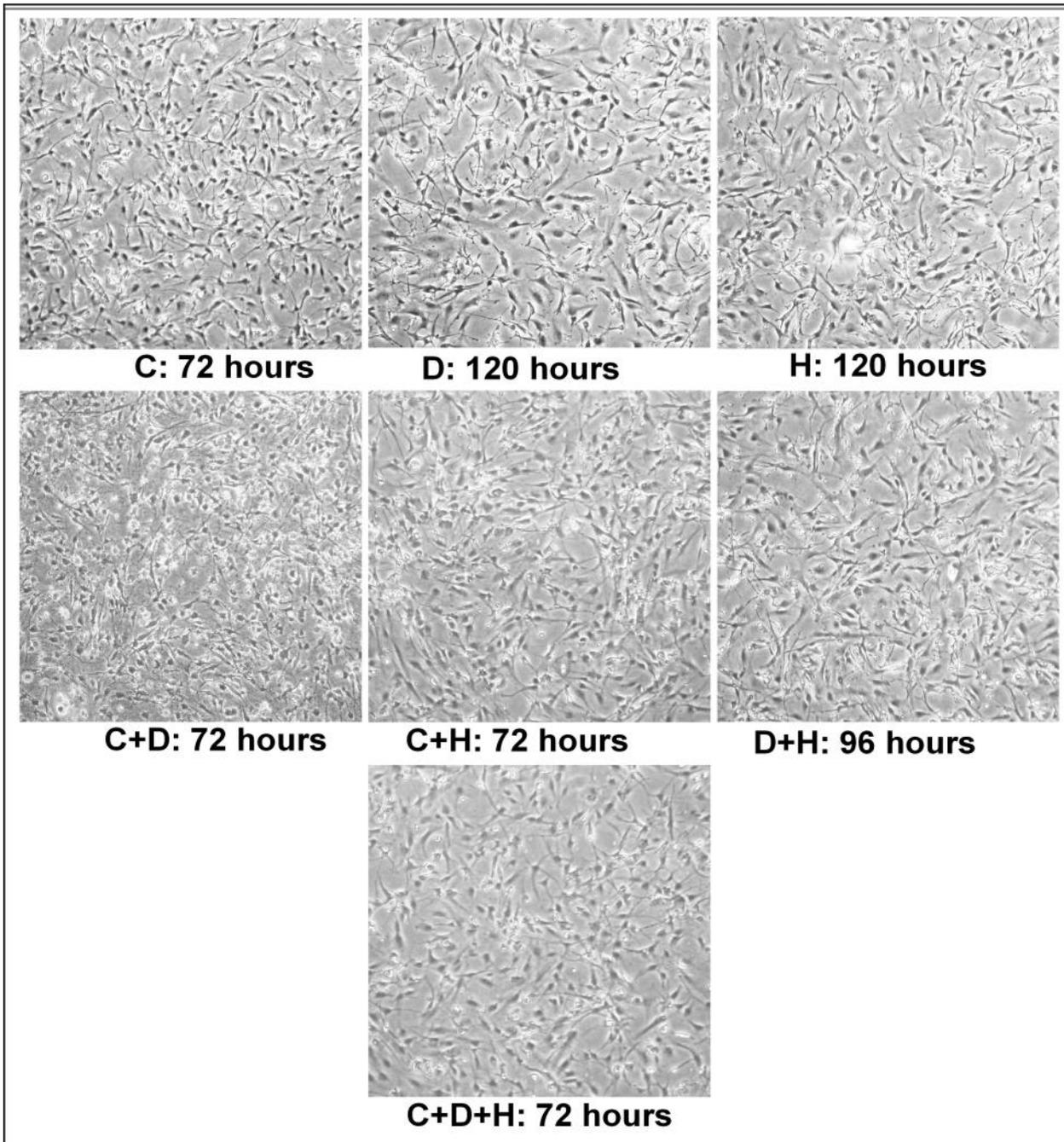
Cells dissociated from most of all conditions were able to grow to 80% confluency within 72 hours (C, C+D, C+H and C+D+H conditions) whereas D+H condition required 96 hours to reach 80% confluency (Figure 5). However, cells dissociated from only 2 conditions, D and H conditions, required 120 hours to grow to 80% confluency.

### Discussion

A pediatric brain tumor is the second most common cancer after leukemia and it is the most common solid cancer occurring in children. Multidrug resistance of children brain tumor to the conventional therapy; chemotherapy and radiation, is still a problematic issue worldwide. Unfortunately, molecular and cellular physiology and pathology of the brain tumor are still very limited, at least in part, due to lack of well-established primary tumor cell lines. Many lines of evidence indicate that the efficiency of many tumor cell line establishment mainly depends on both tumor types and enzyme cocktail conditions<sup>8-9</sup>. However, the present

data indicate for the first time that at least 7 enzyme cocktail conditions commonly used in Thailand provide a similar high effectiveness for establishment of brain tumor cell lines derived from pilomyxoid astrocytoma patients. In addition, different enzyme cocktail conditions still displays some degrees of different effectiveness at this high end.

A previous study using mechanical dissociation established primary cell lines, which were derived from brain tumor tissues. They revealed that the success rate of primary cell line establishment is incredibly low (8.05%)<sup>8</sup>. Therefore, mechanical dissociation technique might be too harsh in disaggregating brain tumor cells for primary cell line establishment resulting in an unsuccessful recovery of the majority of brain tumor cells for *in vitro* growing. Consequently, only small proportion of cells is able to successfully grow in *in vitro* condition leading to a low success rate of establishment. Conversely, enzymatic dissociation technique provides higher success rate (approximately 85%) in establishing primary cell lines compared to the mechanical method<sup>7,9</sup>. However, some research centers prefer mechanical dissociation for establishment. For enzymatic dissociation method, each research center uses different enzyme cocktail components. Ali-Osman



**Figure 5** The morphologies of cells after growing to 80% confluency from 7 enzyme cocktail conditions. This figure shows 80% confluency of cells dissociated from all conditions captured under the light microscope with 32x magnification. Cells dissociated from 4 conditions (C, C+D, C+H and C+D+H) required 72 hours to grow to 80% confluency while D+H conditions required 96 hours for reaching 80% confluency. Only cells dissociated from both D and H conditions required 120 hours to reach 80% confluency (C; collagenase, H; hyaluronidase and D; DNase I).

**Table 1** Summary of different enzyme conditions used for primary cell line establishment<sup>10, 11</sup>

Types of cells	Enzymes used for cell dissociations	Enzyme concentrations	Incubation time
Human mesenchymal from umbilical cord	C+Trp	1 mg/ml + 2.5 mg/ml	3 hours
	C+Trp+H	1 mg/ml + 0.2 mg/ml + 300 ๓g/ml	2 hours
	Trp	2.5 mg/ml (Trp) + 0.2 mg/ml (EDTA)	30 minutes
Pediatric brain tumor	P	200 U/ml	Over night

C: Collagenase; Trp: Trypsin-EDTA; H: Hyaluronidase; P: Papain

**Table 2** The details of brain tumor tissues used for establishing primary cell line

Gender	Female
Diagnosis	Pilomyxoid astrocytomas
WHO grading	IV
Tumor location	Pineal gland
Primary or recurrent tumor	Primary tumor
Surgical resection	Partial resection
Immunohistochemistry	- Section shows necrosis with hemorrhage and increased glial cell proliferation, which are immune stained positive with GFAP, vimentin and S100-protein; focal positive with NSE. - Stains for chromogranin A, synaptophysin, desmin, CD34, panCK(AE1/AE3), EMA, alpha-fetoprotein, PLAP, bHCG, and myogenin are negative. - Ki67 stain for proliferation index of glial cells is positive about 10%.
Treatment (after surgery)	CTX: Vincristin XRT : Cranial : Spine
Status	Alive

**Table 3** Seven enzyme cocktail conditions used for primary cell line establishment

Enzymes	Concentration of enzyme cocktails in 5 ml (U/ml)						
	C condition	D condition	H condition	C+D condition	C+H condition	D+H condition	C+D+H condition
Collagenase type I (Sigma, C0130)	100	-	-	100	100	-	100
DNase type I(Sigma, D4527)	-	100	-	100	-	100	100
Hyaluronidase grade II(Applichem, 373-26-33-3)	-	-	100	-	100	100	100

and colleagues successfully established primary cell lines from normal brain tissues and malignant brain tumor tissues using several enzyme components containing DNase type IA, neutral proteinase and collagenase<sup>7</sup>. Additionally, Rutka and colleagues dissociated tumor tissues obtained from human

gliosarcoma using different enzyme components comprising proteinase, collagenase and DNase<sup>12</sup>. Both studies used different enzyme components to disaggregate tumor tissues into single cells, in addition, both of them successfully established primary cell lines

with high success rate. However, some studies used only 1 enzyme to dissociate cells for cell line establishment. Aoki used proteinase-P solution for cell dissociation<sup>14</sup>. While, Agamanolis used 200U/ml of collagenase for cell dissociation and tissues were incubated over night<sup>11</sup>. This study explored the quality of cell line that was established from pilomyxoid astrocytoma tumor specimen by using 7 different enzyme cocktail conditions. The quality of cell lines established from human brain tissues is very important because these cell lines will be the representatives for further studies of human brain tumors.

Therefore, this study interested in examining the quality of primary cell lines using different enzyme cocktail conditions. The quality of cell lines was determined after cells dissociated with different enzyme cocktail conditions. This study attempted to investigate how well primary cell lines start growing in *in vitro* cell culture condition. The results of cell dissociation revealed that all 7 enzyme cocktail conditions provided similarly good cell dissociation (Figure 1). Brain tumor tissues were generally disaggregated into single cells with some clump cells (Figure 2); hence, numbers of enzymes used for cell dissociation did not significantly affect the degrees of cell dissociation and all conditions produced a similar degree of cell dissociation. The clump cell sizes consistently depend on the number of enzymes used for disaggregating. Bigger sizes of clump cells were found in fewer enzymes used whereas several enzymes used provided smaller sized of clump cells (Figure 2). This indicated that only single enzyme is efficient enough to disaggregate cells into single cells for primary cell line establishment.

Other aspects were also investigated to find the appropriate condition that has less harmful effects on the quality of cell lines. Additionally, the percentages of live cells were also determined to evaluate the toxic effects of enzymes on cell membranes. Surprisingly, all 7 conditions provided similar proportions of live cells (approximately 79%) (Figure 3). Even if 3 enzymes were used to dissociate cells, the percentage of live cells was as high as using single enzyme (Figure 3). Therefore, brain tumor cells were not physically affected on cell

membrane when several enzymes used for cell dissociation. This might be because the concentration of each enzyme used to establish cell lines was low. Consequently, these enzymes did not harmfully destroy plasma membrane of brain tumor cells. Based on the results of cell dissociation, average clump cell size and the percentage of live cells, these indicate that all of 7 enzyme cocktail conditions are not physically harmful to brain tumor cells.

To evaluate the functional aspects of dissociated cells, rate of cell proliferation and time of cell to grow to 80% confluency were performed. Most of 7 enzyme cocktail conditions were successfully proliferate to have  $1.5 \times 10^5$  cells within 120 hours after seeding (Figure 4). However, some conditions (D, H and C+D conditions) required time more than 120 hours. Additionally, all of 7 conditions were able to grow and reach 80% confluency within 120 hours after cell seeding (Figure 5). The previous study, which determined mesenchymal cells isolated from human umbilical cord Wharton's jelly in aspect of the ability of cell growth to 80% confluency. This study plated  $1 \times 10^4$  cells/ml into each well of 96-well plates then they determined the time of cells reaching 80% confluency. The results found that the interval of primary culture and the first passage was 10 days in collagenase with trypsin (C+Trp) condition whereas collagenase with trypsin and hyaluronidase (C+Trp+H) condition had reached 80% confluency on 20 days. Additionally, cells from Trp condition could not continuously grow to 80% confluency after 30 days of seeding<sup>10</sup>. The details of enzyme concentrations of this study are shown in Table 1. Results from the previous study showed that cells isolated by C+Trp had reached 80% confluency faster than C+Trp+H condition<sup>10</sup>. Hence, this probably indicated that more enzymes used might harmfully affect cells compared to less enzymes used but less enzyme used might not effective enough to dissociate cells into single cells and this will affect on cell proliferation. However, the present results showed that all 7 conditions can successfully proliferate and reach 80% confluence. Therefore, these 7 conditions are effective enough to disaggregate cells into single cells and gentle enough for cells to recovery and grow well

after cell dissociation. The concentration of each enzyme used for establishment is as low as 100 U/ml. All enzymes selected to use in this study are useful and provide the advantages for cell dissociation. Several enzymes are required to use in cell dissociation because each enzyme provides different specific effects on cells. The function of collagenase is hydrolyzation of collagen at multiple sites to produce a mixture of small, dialyzable peptides. The collagenase also attacks native collagen and reticular fibers, additionally, it can hydrolyze the other proteins, polysaccharides and lipid in extracellular matrix of connective and epithelial tissues<sup>15</sup>. DNase I is an endoneuclease, which has ability of lysing cells to reduce the viscosity resulting in releasing DNA from damaged cells during cell dissociation process. Additionally, DNase I can also digests double-stranded DNA and high concentration of DNase I can also digest single stranded DNA<sup>16</sup>. In tissue dissociation, DNase I is used during tissue disaggregation to affect on unwanted cell clumping. During the process, cells might be accidentally ruptured resulting in releasing DNA. Therefore, DNase I is used to lyse unwanted DNA released during the process of dissociation<sup>17</sup>. Hyaluronidase is a polysaccharidase, which can cleavage endo-Nacetyl hexosaminic bonds between 2-acetoamido-2-deoxy-beta-D-glucose and D-glucuronate. Hyaluronidase is often used in combination with a crude protease such as collagenase for dissociation of connective tissues<sup>18</sup>. All enzymes have the effects on degradation of the extracellular matrix and disintegration of cell membranes. These capabilities of enzymes might lead to cellular damages when several enzymes are simultaneously used in cell dissociation for primary cell line establishment, especially, when high concentrations of enzymes are used. However, this study revealed that low concentration of enzymes use can avoid this unfavourable effects even several enzymes are used. All enzyme cocktail conditions still effectively preserved the ability of cells to proliferate in *in vitro* condition after cell dissociation.

## Conclusion

This study revealed that several enzymes used for cell dissociation did not physically affect cell structures compared to single enzyme used. Therefore, single enzyme used for cell dissociation in primary cell line establishment might be efficiently enough to establish cell lines from brain tumor tissues derived from pilomyxoid astrocytoma patient.

## Limitations of this study

To establish primary cell line derived from patients, brain tumor tissues removed from patients should be processed to establish cultured cells as soon as possible to obtain the highest yield of establishment. Therefore, the experiments were repeated twice or triplicate during the establishment and they could not be independently repeated.

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