中 國 醫 藥 大 學
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碩士學位論文

Micro-RNAs 在惡性神經膠質母細胞瘤
與正常腦細胞中之不同表現

Micro-RNAs Express Differentially in Glioblastoma Multiforme and Normal Brain Cells

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中文摘要

試驗主題

　　本研究主要是研究惡性神經膠質瘤中，microRNA 於神經腫瘤細胞及神經腫瘤幹細胞中扮演之角色。我們以組織庫挑選收集病人檢體（已獲得病人同意後），包含惡性神經膠質瘤和正常腦組織檢體細胞，篩檢出有意義的 micro-RNA，利用 GBM tissue microRNA Chip Data Analysis 瞭解惡性神經膠質瘤和正常腦組織細胞中 microRNA 之表現及差異程度。

研究背景及目的

　　即使最先進之治療改變包括先進手術導航手術方法，化學治療，放射線治療， 對 於惡性神經膠質瘤之預後，進展仍十分有限。只能多延長 3 至 6 個月生命，腫瘤幹細胞雖然只佔 1-2%之惡性腫瘤，它卻有堅強生長分化能力，且對臨床之治療有頑強之抗性。它具有強大自我更新及 DNA 修護能力，我們認為 microRNA 在神經腫瘤細胞及神經腫瘤幹細胞中之角色扮演極為重要，有急需研究瞭解之必要。目前已知在不同狀態下的神經腫瘤細胞及神經腫瘤幹細胞(CD133$^+$, CD133$^-$)之中，會有不同的 microRNA 表現，如能知道 microRNA 影響神經腫瘤細胞生長分化因子，就可以擬定治療改進之對策，以增進惡

性神經膠質瘤預後。

實驗設計

本實驗以組織庫挑選收集病人檢體((檢體 6 件，實驗檢體取自本院組織庫中已獲得病人同意之檢體，包含惡性神經膠質瘤和正常腦組織等四大類的檢體。)

，包含惡性神經膠質瘤和正常腦組織檢體，利用 GBM tissue microRNA Chip Data Analysis 瞭解惡性神經膠質瘤和正常腦組織細胞中 microRNA 之表現及差異程度。

我們統計並比較 microRNA 在不同檢體中的表現差異(miRNA intensity<1, adjust to 1, Ratio=log2（case#1/case#2）)，此外我們也利用分析出來的資料，利用 Gene expression assay datas of Normal Brain Tissue（GDS596）做基因庫的分析（from Affymetrix U133A and GBM primary culture cell lines (Affymetrix U133 Plus 2.0)). 我們發現有些人類細胞或病毒株之 microRNA 具有相當意義之正調控或負調控的表現，而這些具統計意義之表現存在於惡性神經膠質瘤和正常腦組織細胞之中.

結論

　　我們可以初步證實 microRNA 在惡性腦瘤幹細胞中扮演的角色，並期待 microRNA 正調控或負調控的表現，可以影響惡性神經膠質瘤細胞之表現，並期待可以發展可行之治療策略，以增進治療惡性神經膠質瘤之臨床治療預後。

關鍵詞：神經腫瘤幹細胞、惡性神經膠質瘤、免疫細胞治療、 Gene expression assay、microRNA、 microRNA Chip Data Analysis。

Abstract

Object

We hypothesize that Micro-RNAs might play an important roles in glioblastoma glastoma (GBM) cells, and differentially express between GBM and normal brain cells. We compare clinical datas using MicroRNA Chip Data Analysis and statistical analyses by use of the algorithm in miRBase.

Methods

We compare clinical datas using MicroRNA Chip Data Analysis and statistical analyses by use of the algorithm in miRBase. After surgical resection, we make definite GBM pathological specimen examination and to culture GBM cells. The normal brain tissue was donated from a patient expired with intracranial lesions, and we excise the normal brain tissue within 30 minutes just after his death.

We want to find some differently expressed miRs (If miRNA intensity<1, adjust to 1, Ratio=log2 (case#1/case#2)) in these 4 types of cells, and adopt to molecular- biological research for malignant brain tumors. Furthermore, we also adopt our preliminary datas to compare with Gene expression assay datas of Normal Brain Tissue (GDS596) from Affymetrix U133A and GBM primary culture cell lines (Affymetrix

U133 Plus 2.0)

Results

We have obtained a promising preliminary miRNA profiling which resulted from comparing with GBM and non-tumor brain tissues. By use of the algorithm in miRBase Targets, some miRNAs have been computationally predicted to show the significantly change of expression ratio in our preliminary miRNA data.

Conclusion

Some Micro-RNAs significantly expressed differentially in GBM cells and normal brain cells. Some express as up-regulation, and others express as down-regulation. Further study including Micro-RNAs RT-PCR and gene expression assay are ongoing. Micro-RNAs might play an important

Keyword: Astrocytoma, Gene expression assay, GBM, Glioblastoma multiforme, Micro-RNA, Micro-array, micro-RNA chip.

致謝

　　首先得感謝科內及醫院支持我進修, 這兩年來因為學業的關係不無擔擱了許多臨床工作, 感謝其他科內先進同事不吝給于支持和幫助, 才得以安心於學業.

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　　最後要把我的學位和論文獻給天上的父親,謝謝您.

目次

第一章 Background（前言及研究背景）

The prevalence of malignant brain tumor of the world is 4-5 cases per 100000 annually. For the world population more than 60 billion, about 66000 new malignant brain tumor cases appear in every year. Although the number is limited, the mortality rate is contracted higher than other cancers. In all neurosurgeons' clinical experience, all of these patients suffer from inconveniently neurological deficit and mostly die within one year (6-8 months) after initial treatment. Despite of the development of high technology, such as operative navigation system, advanced neuroimaging of functional MRI and diffusion tensor imaging, more precisely radiosurgery of Gamma knife or Cyberknife, more effective chemotherapy of Temozolamide or gliadal wafer, the prognosis is still miserable for prolong just a short interval (3-6 months) for life span. [5]

Human glioblastoma multiforme (GBM) exhibits its highest incidence about 65 years of life and hence is a disease of aging. Despite intensive effort to devise new therapies, GBM remain rapidly and uniformly mortal, particularly in the aged population. Some patients' GBM respond to chemo- and radio- therapy and some don't, and it is believed that cancer stem cells may be the cause. Stem cells — popularly known as a source of biological rejuvenation — may play harmful roles in the body, specifically in the growth and spread of cancer. Among the wildly dividing cells of a tumor, scientists have located cancer stem cells. Stem cell-like glioma cancer cells that share many characteristics with normal stem cells propel the lethal growth of brain cancers by promoting

tumor blood vessel formation, and may hold the key to treating these lethal cancers [5].

MicroRNAs (miRNAs) are small (19–25 nucleotides) endogenous noncoding RNAs that have been shown to influence the abundance and translational efficiency of cognate mRNAs, and to control critical time points in development of plants and animals. In many published literatures, miRNAs can function either as oncogenes or as tumor suppressors in their effect on tumor growth, including Gliomas. For example, miR-7 and miR-21 were reported as implicating in GBM by comparing miRNAs expression in tumor and normal tissue [1,8]. Alterations of several miRNAs have been linked to cancer development and its biology. Recent study has been shown that loss of miRNA expression might contribute to upregulate the telomerase protein. [15].
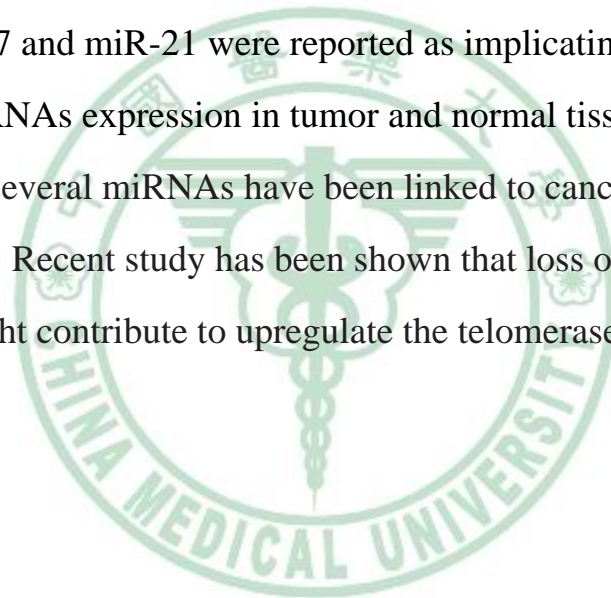
第二章 Methods and Materials
（ 研究材料與研究設計 ）

We hypothesize that Micro-RNAs might play an important roles in glioblastoma glastoma (GBM) cells, and differentially express between GBM and normal brain cells.

We compare clinical datas using MicroRNA Chip Data Analysis and statistical analyses by use of the algorithm in miRBase. After surgical resection, we make definite GBM pathological specimen examination and to culture GBM cells. The normal brain tissue was donated from a patient expired with intracranial lesions, and we excise the normal brain tissue within 30 minutes just after his death.

We use GBM tissue microRNA Chip Data Analysis (Agilant) to examine the microRNA profiles. Totally 5 GBMs (GBM #1 - #5) and 1 normal brain tissue was done, but GBM #2 and GBM #5 was failed due to poor quality of microRNA profiles . (Figure 1)

We assign the samples number as:
- GBM patient #1
- GBM patient #3
- GBM patient #4
- Normal Brain tissue (Control)

GBM tissue microRNA Chip Data Analysis (Agilant) include probeset of 799 miRNA, including:

- Ebv-miR (Epstein Barr virus miRNA): 39

- cmv-miR (Human cytomegalovirus miRNA): 17

- Hsa-miR (Homo Sapiens miRNA): 723

- Hiv1-miR (Human immunodeficiency virus miRNA): 2

- Hsv1-miR (Herpes Simplex Virus 1 miRNA): 2

- Kshv-miR (Kaposi sarcoma-associated herpesvirus miRNA):

We want to find some differently expressed miRs (If miRNA intensity<1, adjust to 1, Ratio=log2 (case#1/case#2)) in these 4 types of cells, and adopt to molecular- biological research for malignant brain tumors. Furthermore, we also adopt our preliminary datas to compare with Gene expression assay datas of Normal Brain Tissue (GDS596) from Affymetrix U133A and GBM primary culture cell lines (Affymetrix U133 Plus 2.0)

We have obtained a promising preliminary miRNA profiling which resulted from comparing with GBM and non-tumor brain tissues. By use of the algorithm in miRBase Targets, some miRNAs have been computationally predicted to show the significantly change of expression ratio in our preliminary miRNA data.

第三章 Results（ 研究結果 ）

miRNA Expression Profiles

We use mirBase If miRNA intensity<1, adjust to 1.Ratio=$\log_2$ (case#1/case#2). All of miRNAs Expression Profiles, 322 miRNAs flaged (Ratio>0), 122 Significant Expressed miRNAs (P<0.005) (Figure 2-1, 2-2, 2-3)

According to the primary datas on micro-RNAs chip, we correct and analyze the expression ratio as 2 folds changed expression in all condition, and we limit the miRNAs as has-miR-96, has-miR-630, has-miR-435-5P, has-miR-150, has-miR-887, has-miR-513-c, has-miR-923, has-miR-135a, has-miR-513a-5p, has-miR-513b, hsv1-miR-H1, hcmv-miR-UL70-3p. (Figure 3-1)

There were 385 miRNAs of 2-folds-change Referenced by Normal Brain (Ratio>=2-folds in condition 1-3) (Figure 3-2).

We also analyze Brain-specific miRNA (miR-129, miR-219, miR-330, miR-149, miR-153,miR-181a, miR-221) Expression Profile in GBM miRNA Chips (Figure 3-3). 94 miRNAs are noticed as 2-folds change reference by normal brain tissue (Figure 3-4).

Cluster of miRNA expression profiles

We make cluster analyses, and gain the results followed:

■ Filter out intensity<=1: **448 miRNAs** (Figure 4-1)

- Intensity>=1 and ratio>=1 in any GBM referenced by normal: **390 miRNAs** (Figure 4-2)

- Intensity>=1 and Flag='P'or Flag≠'A' in all conditions and ratio>=1 in any GBM referenced by norma: **295 miRNAs** (Figure 4-3)**.**

Virus GBM miRNA Expression Profiles (76 virus mi-RNAs)

We also performed Virus GBM miRNAs Expression Profiles (76 virus mi-RNAs). We collect the datas of 2-folds change in any condition, and try to define which are   up-regulated mi-RNAs and which are down-regulated ones.(Figure 5-1)

Differentially Expressed miRNA

Finally, we can get datas of mi-RNAs (from human or virus) of differentially significant expression in GBM cells and normal brain cells, including up-regulated mi-RNAs and down-regulated ones (Figure 5-2).

In comparison of GDS596 Normal brain tissue gene expression assay datas

We adopt our preliminary datas to compare with Gene expression assay datas of Normal Brain Tissue (GDS596) from Affymetrix U133A and GBM primary culture cell lines (Affymetrix U133 Plus 2.0) (Table 1-1, 1-2)

第四章 Discussion （ 結果討論 ）

MicroRNAs (miRs) are a novel group of short RNAs, 22 nucleotide in length, that regulate gene expression in a posttranscriptional manner by pairing with complementary nucleotide sequences in 3' untranslatable region of target mRNA. Abnormal expression of miRs is linked with various human disorders [3,13,17].

In many published literatures, miRNAs can function either as oncogenes or as tumor suppressors in their effect on tumor growth, including Gliomas. For example, miR-7 and miR-21 were reported as implicating in GBM by comparing miR expression in tumor and normal tissue.[3,6]. miRNA-21 is known to be an antiapoptotic factor in human glioblastoma cells, not only in vitro but also in vivo studies[3,4]. MicroRNA-7 has been reported to inhibis the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma [2]

Chemotherapy agents and mi-RNAs

Temozolamide and gliadel wafer have been widely used as first line chemotherapy for malignant brain tumors, but the prognosis is still miserable for those efforts. The research team of Professor Hilah had reported that transfection of miR-451 combined with Imatinib mesylate treatment had a cooperative effect in dispersal of GBM neurospheres. In addition, Identification of additional miRs and target genes that regulate GBM stem cells may provide new potential drugs for therapy. [9,10]

Genes can be regulated by miRNAs

MicroRNAs (miRNAs) get involved in critical biological processes by repressing the translation of coding genes. Previous study finds that more than one-third of human genome are regulated by RNA. Thousands of human genes are microRNA targets [11,14].

miRNA Expression Profiles in Normal Brain Tissues

345 miRNA expression data in 40 normal human tissues [12]. According to the microarray and statistical analysis, we divide micro-RNAs into 3 groups:

1. Absolute tissue-specific miRNAs
   a. miRNA is detectable at sufficient levels in the specific tissue but is undetectable in all or most of the remaining tissues, ex. miR-129, miR-219, miR-330.
   b. miRNA expression is >20-fold higher in the specific tissue(s) compared with the mean of the other tissues, ex. miR-149, miR-153,miR-181a, miR-221
2. Tissue-enriched miRNAs (miRNA expression >5-fold higher in the specific tissue(s) compared with the mean of the other tissues), ex.not found in normal brain tissue

   Most differentially expressed miRNAs in each tissue.

Using targeted mi-RNAs found by published papers in comparison of our samples according to mi-RNAs micro-array profiles

1.   MicroRNA -451

The research team of Professor Hilah examined the microRNA

profiles of Glioblastoma stem (CD133+) and non-stem (CD133⁻) cell populations and found up-regulation of several miRs in the CD133⁻ cells, including miR-451, miR-486, and

miR-425, some of which may be involved in regulation of brain differentiation. They use the above miRs to transfect GBM cells, and the miRs are result in inhibiting and dispersing neurospheres, and inhibiting GBM cell growth [9].

According to our presented data, hsa-miR-425, hsa-miR-425*, hsa-miR-451, hsa-miR-486-5p and hsa-miR-486-3p are expressed as down-regulated (Table-2).

2. MicroRNA- 21

MicroRNA 21 (miR-21) is significantly elevated in glioblastoma (GBM).

Authors demonstrate that miR-21 regulates multiple genes associated with glioma cell apoptosis, migration, and invasiveness. [7]

According to our presented data, hsa-miR-21 is expressed as up-regulated, too.　(Table-3).

3. MicroRNA-124 and microRNA-137

MicroRNA-124 and microRNA-137 induce differentiation of adult mouse neural stem cells, mouse oligodendroglioma-derived stem cells and human glioblastoma multiforme-derived stem cells, and cell cycle in GBM was induced to arrest. MiR-124 and miR-137are found to inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. [16]

According to our presented data, MicroRNA-124 and microRNA-137 express as up-regulated, too. (Table -4).

第五章 Conclusion（ 結論 ）

In our presented data, we can gain some Micro-RNAs significantly expressed differentially in GBM cells and normal brain cells. Some Micro-RNAs express as up-regulation, and others express as down-regulation. Same results have been presented in published literatures. Further study including Micro-RNAs RT- PCR and gene expression assay are ongoing. In our opinions, Micro-RNAs might play an important role in GBM cells. It might have differential expression in GBM compared with normal brain cells, and to identify the functional contribution of key miRNAs in the regulation of GBM cells is necessary.
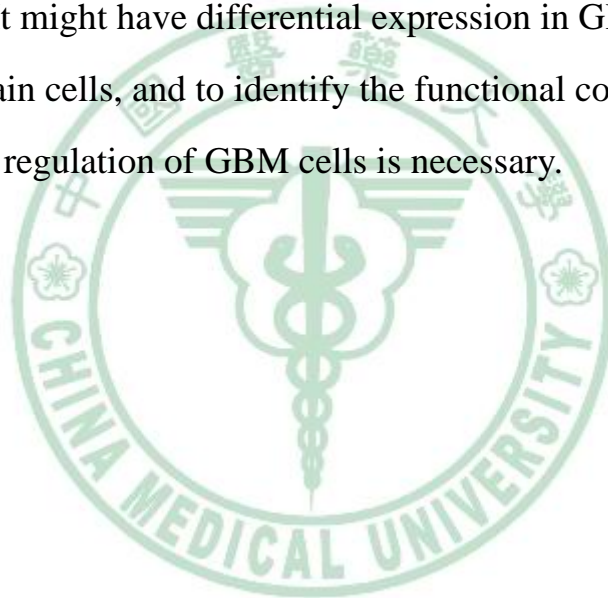
Figure 1. RNA QA / QC information

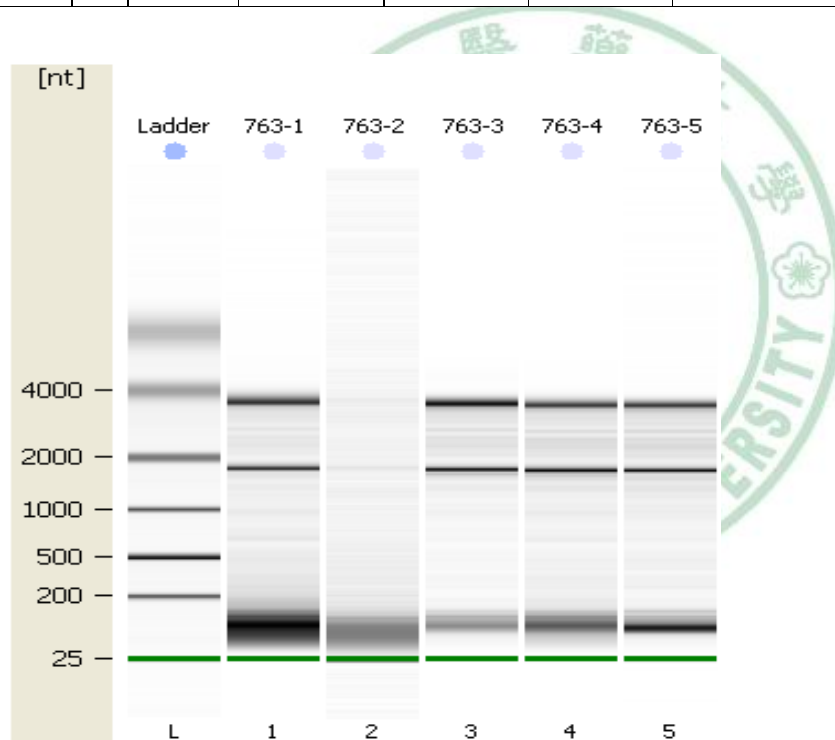|	Sample name	Sample weight (g)	RNA conc. (ng/μl)	RNA quantity (ng)	OD260/280 Ratio	Bioanalyzer chip lane location	28S/18S Ratio	RIN
#1	1	0.08	1059.82	31794.60	1.97	1	2.0	6.5
#2	2	0.09	635.20	19056.00	1.62	2	N/A	2.5
#3	3	0.10	1938.79	58163.70	2.01	3	1.5	8.3
#4	4	0.08	1218.01	36540.30	2	4	1.2	7.5
#5	normal	0.08	286.55	8596.50	1.99	5	1.3	6.9

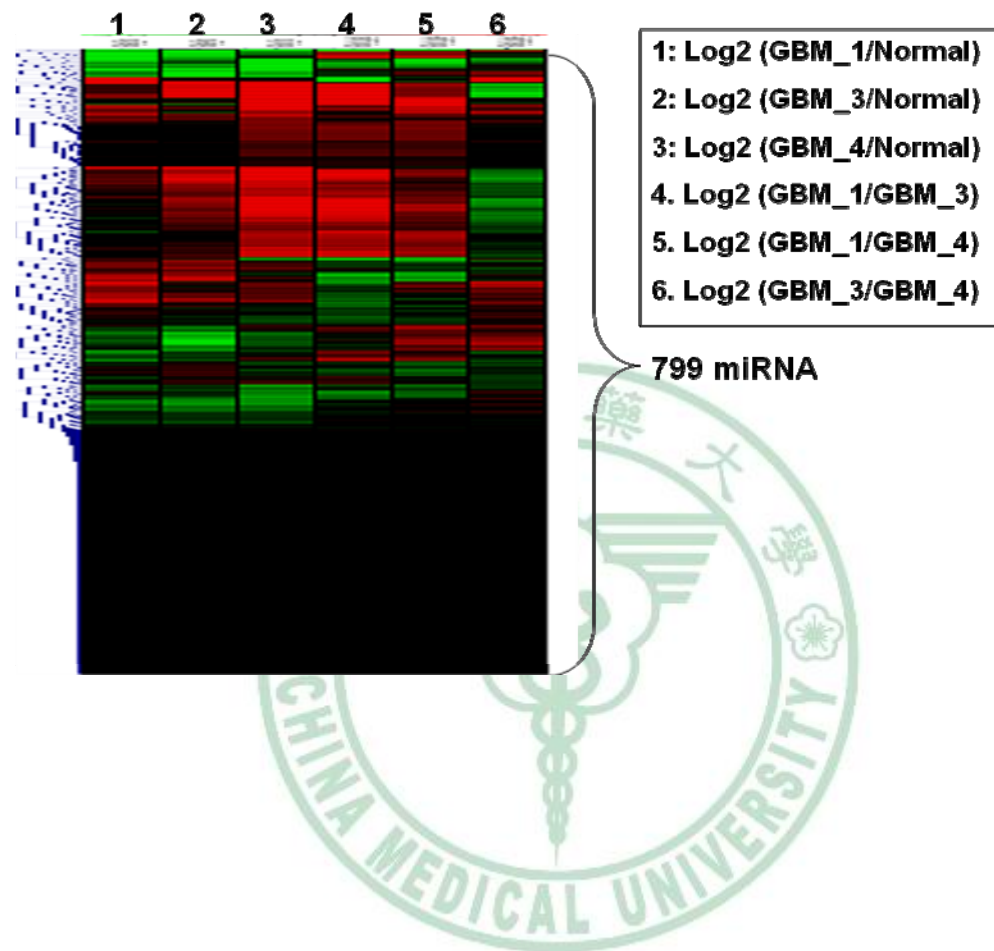# Figure 2-1

(All 799 miRNAs in miRNAs Expression Profiles)

1: Log2 (GBM_1/Normal)

2: Log2 (GBM_3/Normal)

3: Log2 (GBM_4/Normal)

4. Log2 (GBM_1/GBM_3)

5. Log2 (GBM_1/GBM_4)

6. Log2 (GBM_3/GBM_4)

799 miRNA

Figure 2-2

(Represented as 322 miRNAs flaged (Ratio>0))

1: Log2 (GBM_1/Normal)
2: Log2 (GBM_3/Normal)
3: Log2 (GBM_4/Normal)
4. Log2 (GBM_1/GBM_3)
5. Log2 (GBM_1/GBM_4)
6. Log2 (GBM_3/GBM_4)
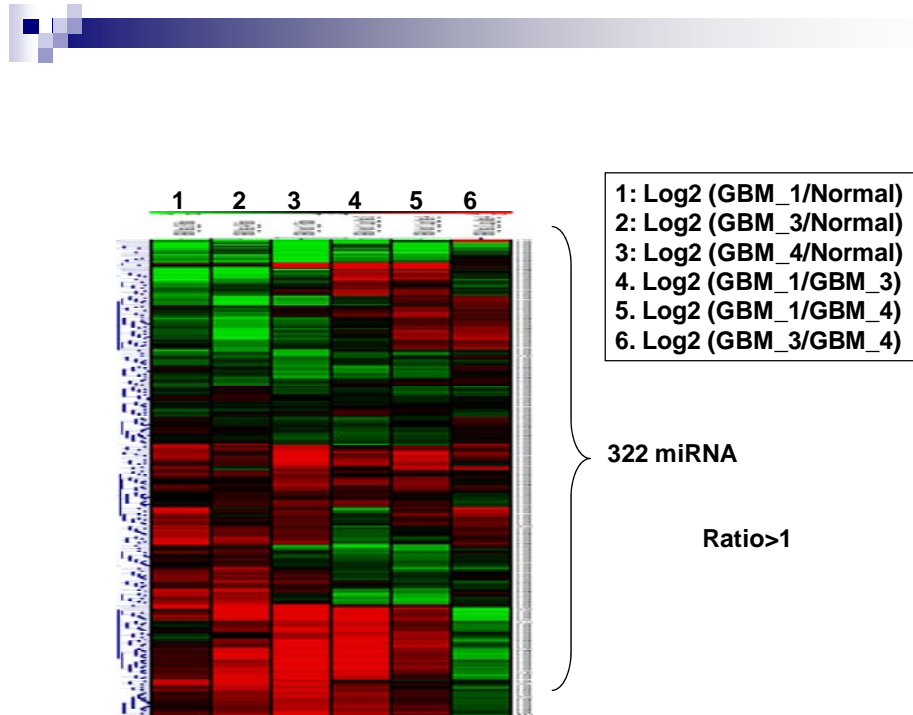
322 miRNA

Ratio>1

Figure 2-3.(Significantly represented as 122 miRNAs flaged

(Ratio>0, P-value <=0.05))

1: Log2 (GBM_1/Normal)
2: Log2 (GBM_3/Normal)
3: Log2 (GBM_4/Normal)
4. Log2 (GBM_1/GBM_3)
5. Log2 (GBM_1/GBM_4)
6. Log2 (GBM_3/GBM_4)

122 miRNAs

P-value<=0.05

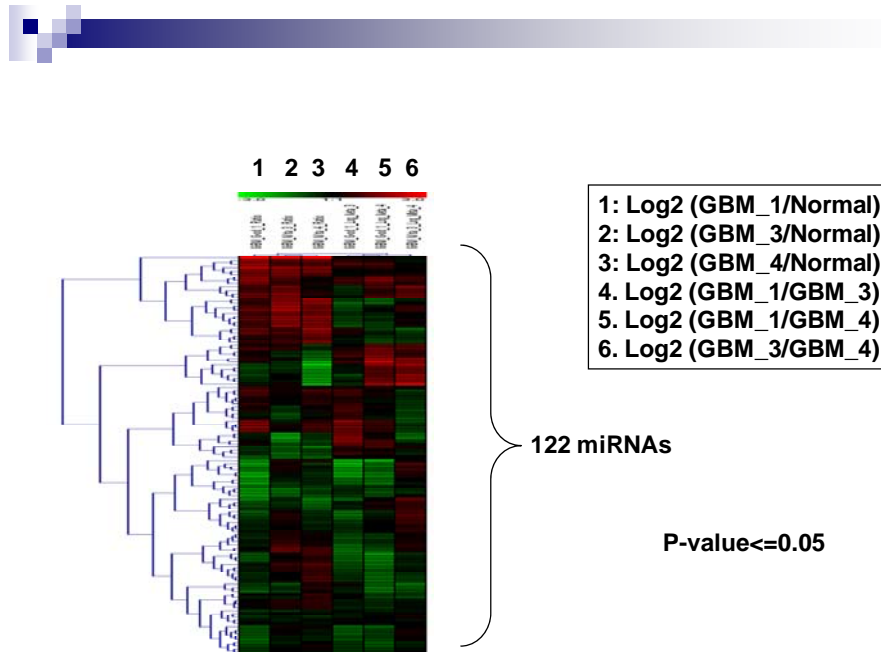# Figure3-1. (2 folds changed expression on miRNA chip in all condition)
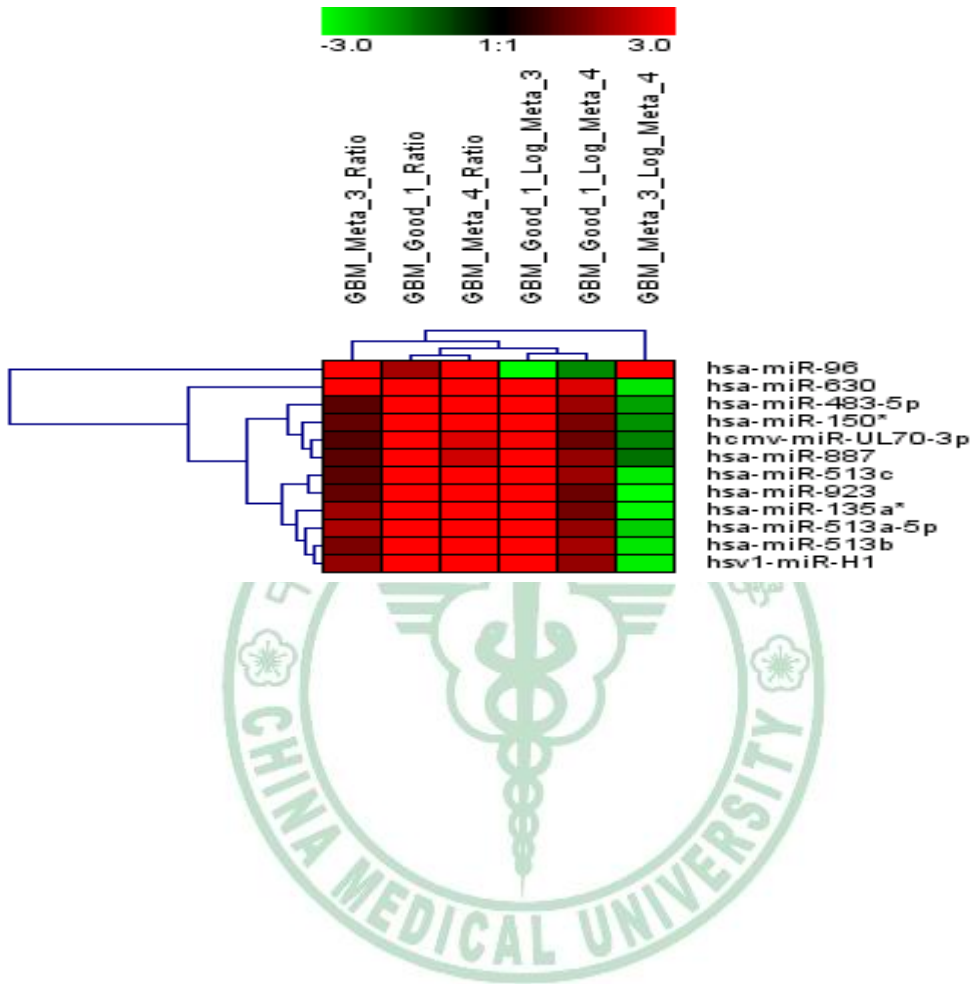
Figure3-2.(2-folds-change Referenced by Normal Brain
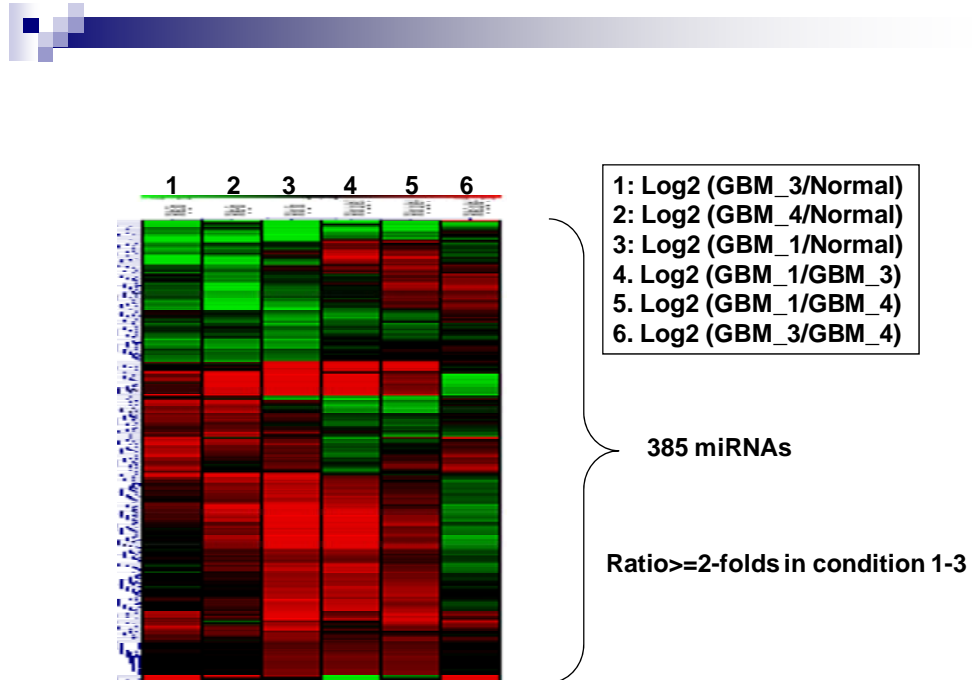
(Ratio>=2-folds in condition 1-3) 385 miRNAs

1: Log2 (GBM_3/Normal)
2: Log2 (GBM_4/Normal)
3: Log2 (GBM_1/Normal)
4. Log2 (GBM_1/GBM_3)
5. Log2 (GBM_1/GBM_4)
6. Log2 (GBM_3/GBM_4)

385 miRNAs

Ratio>=2-folds in condition 1-3

Figure 3-3. Brain-specific miRNA Expression Profile in GBM miRNA Chips

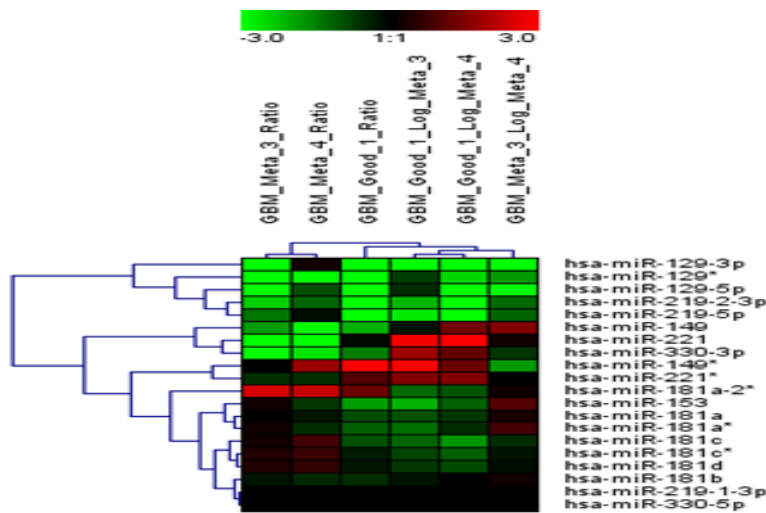| miR-129, miR-219, miR-330, miR-149, miR-153,miR-181a, miR-221	brain

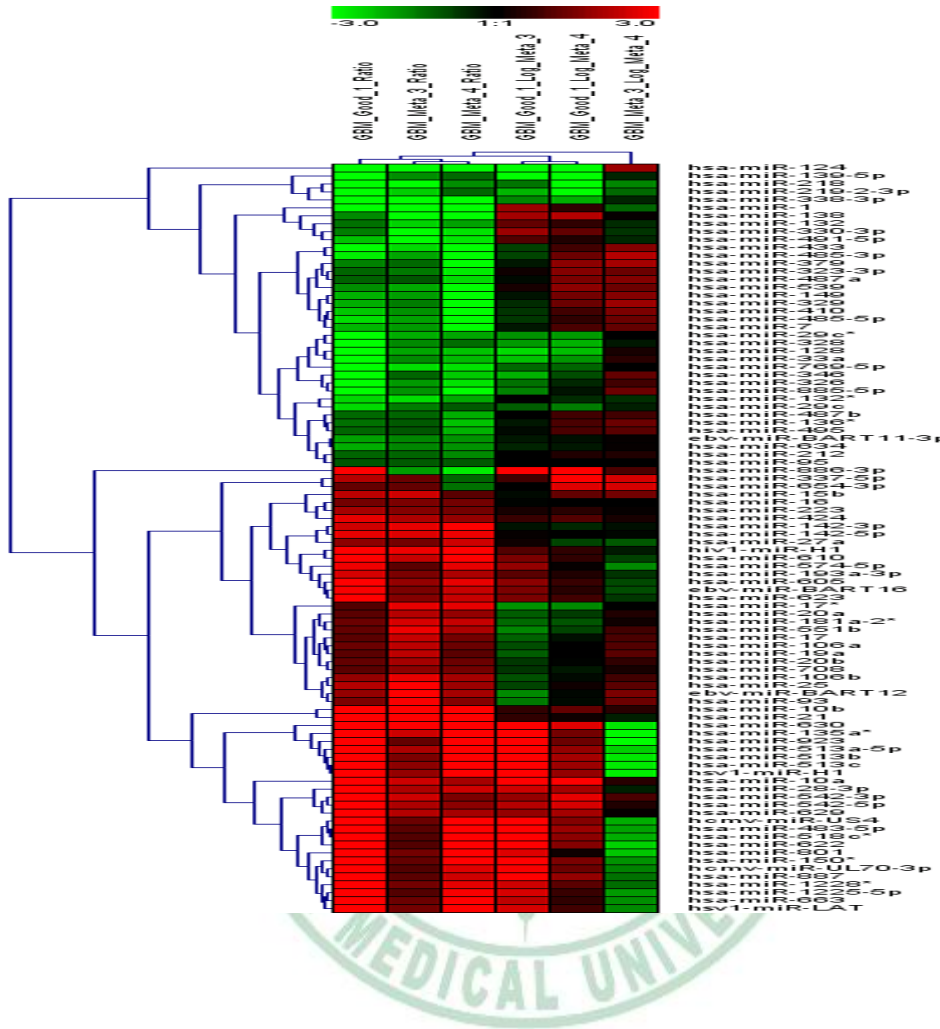# Figure 3-4. 94 miRNAs are noticed as 2-folds change reference by normal brain tissue

Figure 4-1.(Filter out intensity<=1: 448 miRNAs)
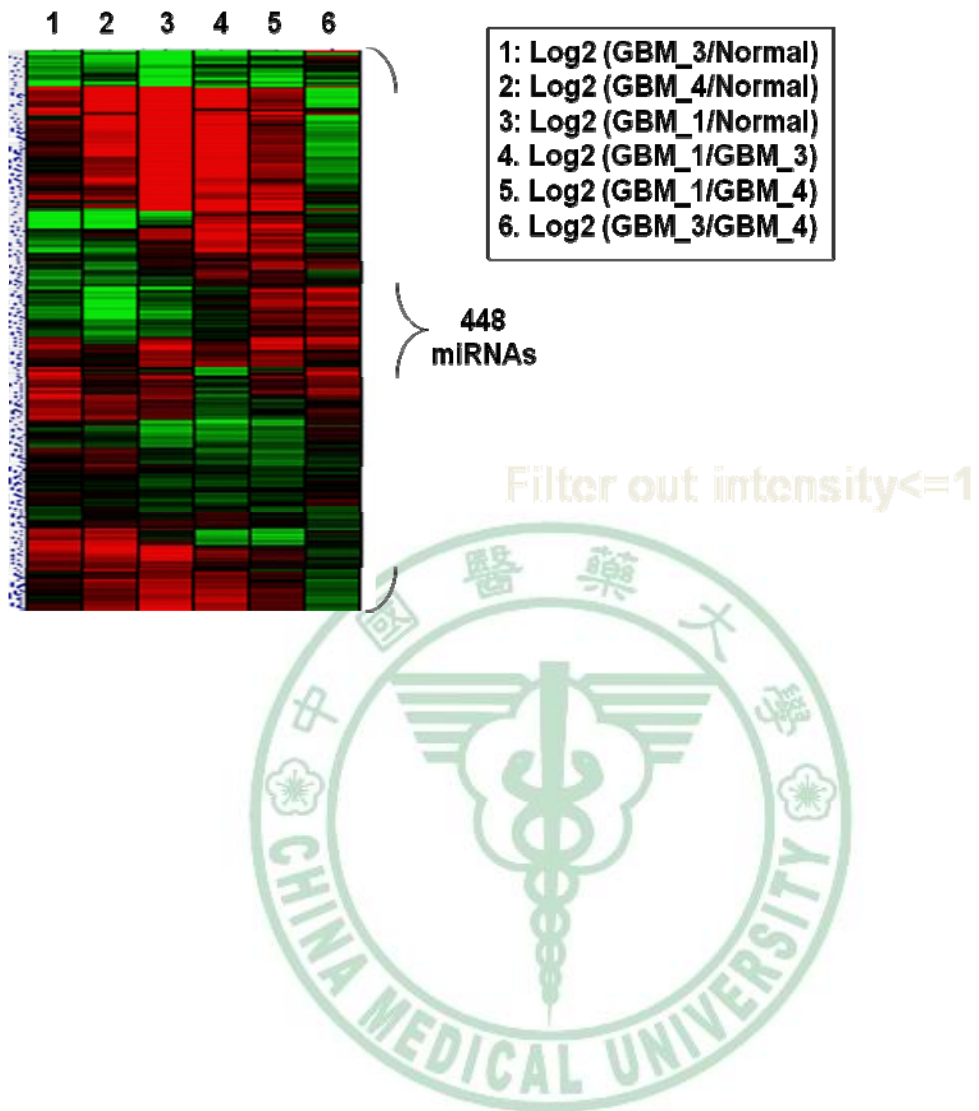

1: Log2 (GBM_3/Normal)
2: Log2 (GBM_4/Normal)
3: Log2 (GBM_1/Normal)
4. Log2 (GBM_1/GBM_3)
5. Log2 (GBM_1/GBM_4)
6. Log2 (GBM_3/GBM_4)

448 miRNAs

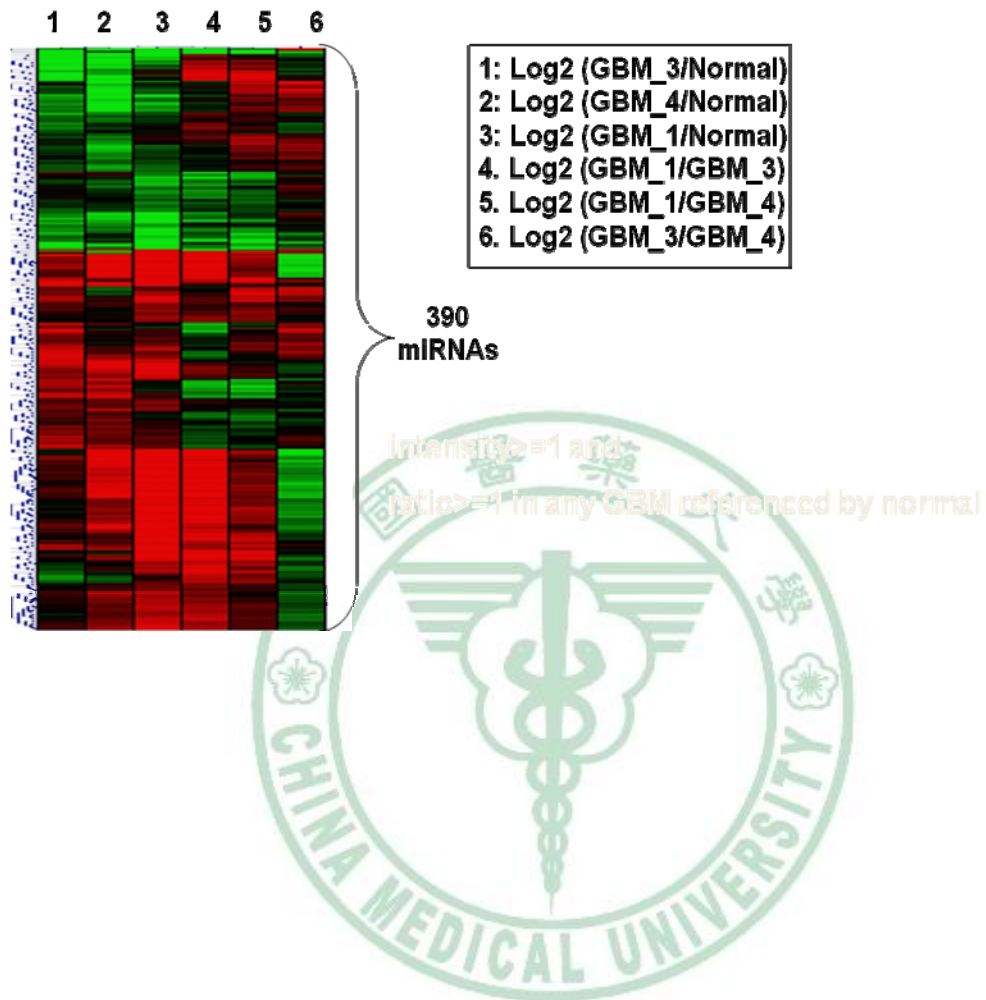# Figure 4-2.(intensity>=1 and ratio>=1 in any GBM referenced by normal: 390 miRNAs)

Figure 4-3. (intensity>=1 and Flag='P, Present' or Flag≠'A, Absent'in all conditions and ratio>=1 in any GBM referenced by norma: 295 miRNAs)

1: Log2 (GBM_3/Normal)
2: Log2 (GBM_4/Normal)
3: Log2 (GBM_1/Normal)
4. Log2 (GBM_1/GBM_3)
5. Log2 (GBM_1/GBM_4)
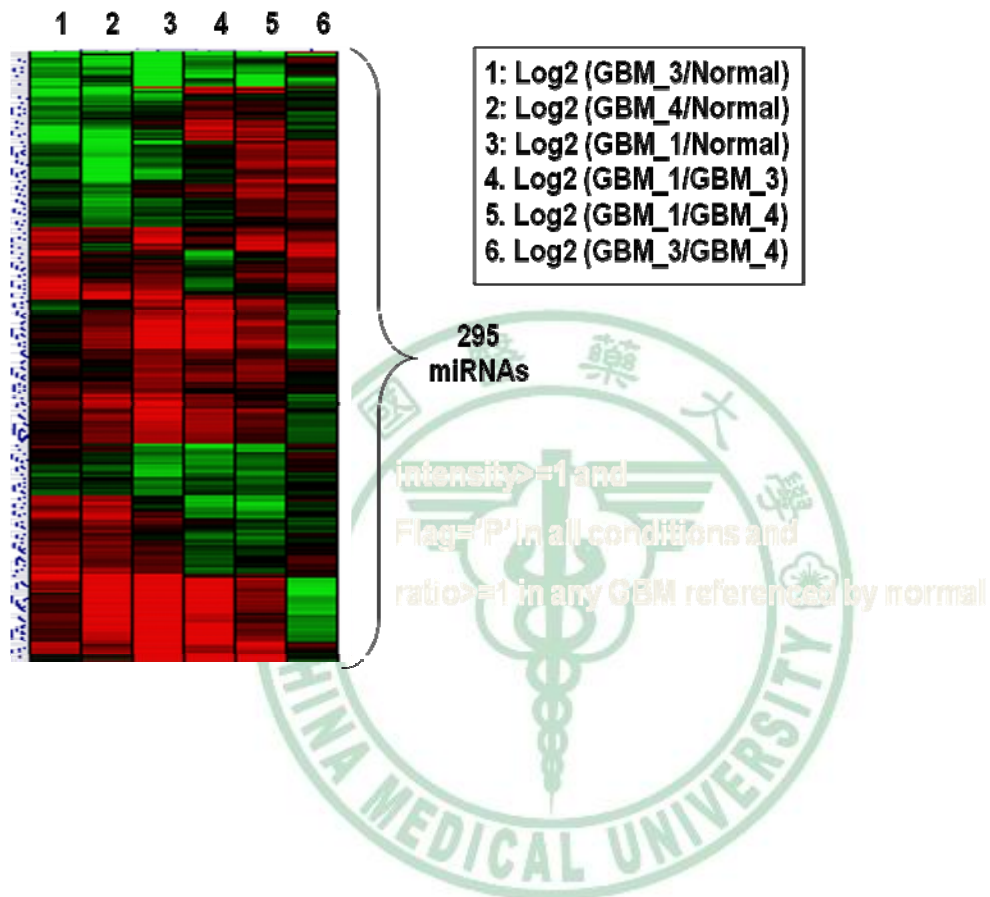6. Log2 (GBM_3/GBM_4)

295 miRNAs

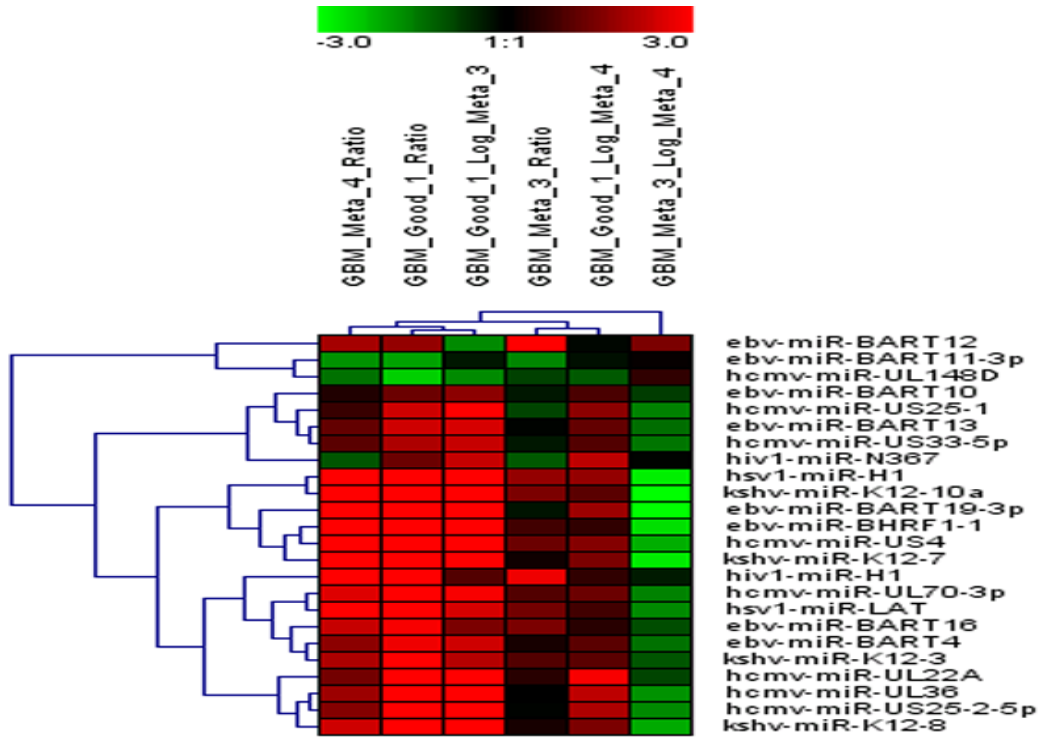# Figure 5-1. Virus GBM miRNA Expression Profiles (76 virus mi-RNAs)

Figure 5-2. Differentially Expressed miRNA (Up-regulation: green, down-regulation: red.)

Condition	fold	Human (Up/Down)	Virus (Up/Down)	Total (Up/Down)
All condition	>=4	37 (22/15)	3 (up)	40 (10/6)

miRNA_ID
hsa-miR-1
hsa-miR-124
hsa-miR-129*
hsa-miR-133a
hsa-miR-133b
hsa-miR-29b-2*
hsa-miR-31
hsa-miR-338-3p
hsa-miR-377*
hsa-miR-433
hsa-miR-491-5p
hsa-miR-519d
hsa-miR-577
hsa-miR-769-5p
hsa-miR-935

miRNA_ID
hsa-miR-195*
hsa-miR-200a*
hsa-miR-200c
hsa-miR-21
hsa-miR-21*
hsa-miR-28-3p
hsa-miR-373*
hsa-miR-424*
hsa-miR-452
hsa-miR-513a-5p
hsa-miR-610
hsa-miR-630
hsa-miR-96

miRNA_ID
ebv-miR-BART1-5p
ebv-miR-BART7
hiv1-miR-H1
hsa-miR-10b
hsa-miR-10b*
hsa-miR-135a*
hsa-miR-142-3p
hsa-miR-142-5p
hsa-miR-182
hsa-miR-183
hsa-miR-183*
hsa-miR-187*
hsa-miR-195*

Table 1-1. In comparison of GDS596 Normal brain tissue gene expression assay datas (All genes: 11,934 [$\log_2$(GBM/Normal)])

Condition	fold	Up-regulation	Down-regulation	Total
Any condition	>=2	2131	6848	8979
Any condition	>=4	815	3782	4597
Any condition	>=6	421	2345	2766
Any condition	>=8	245	1643	1888
All condition	>=2	960	4545	5505
All condition	>=4	308	2065	2373
All condition	>=6	159	1168	1327
All condition	>=8	89	739	828

Table 1-2. In comparison of GDS596 Normal brain tissue

gene expression assay datas (Filters: intensity>=1 and Flag='P')

Condition	fold	Human (Up/Down)	Virus (Up/Down)	Total (Up/Down)
Any condition	>=2	281 (175/106)	14 (12/2)	295 (187/108)
Any condition	>=3	236 (151/85)	14 (12/2)	250 (163/87)
Any condition	>=4	180 (118/62)	13 (12/1)	193 (130/63)
Any condition	>=5	126 (79/47)	10 (10/1)	136 (89/47)
All condition	>=2	86 (46/42)	8 (7/1)	94 (53/41)
All condition	>=3	39 (17/22)	4 (3/1)	43 (20/23)
All condition	>=4	15 (9/6)	1 (up)	16 (10/6)
All condition	>=5	8 (4/5)	1 (up)	9 (5/4)

Table-2. hsa-miR-425, hsa-miR-425*, hsa-miR-451,

hsa-miR-486-5p and hsa-miR-486-3p are expressed as

down-regulated.

miRNA_#	1_Raw	3_Raw	4_Raw	1:3 log2	1:4 log2	3:4 log2
hsa-miR-425	128.538	71.8072	98.4294	0.83999	0.38503	-0.45496
hsa-miR-425*	21.1687	13.7831	20.6556	0.61903	0.0354	-0.58363
hsa-miR-451	45248.7	19364.4	6893.76	1.22447	2.71451	1.490044
hsa-miR-486-5p	272.111	126.482	78.6066	1.10526	1.79147	0.68621
hsa-miR-486-3p	3.88354	1.64041	2.95655	1.24332	0.39346	-0.84986

Table 3. hsa-miR-21 are expressed as up-regulated

miRNA_#	1_Raw	3_Raw	4_Raw	1:3 log2	1:4 log2	3:4 log2
hsa-miR-21	47025.40	30191.40	39717.80	0.6393	0.24365	-0.39565
hsa-miR-21*	144.46	45.95	30.88	1.65258	2.2261	0.57352

Table 4. MicroRNA-124 and microRNA-137 express as up-regulated

miRNA_#	1_Raw	3_Raw	4_Raw	1:3 log2	1:4 log2	3:4 log2
hsa-miR-137	5.64645	127.672	14.2783	-4.499	-1.3384	3.16055
hsa-miR-124	28.4028	2124.61	591.786	-6.225	-4.381	1.84405

第六章 References（ 參考文獻 ）

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