科技部補助

大專學生研究計畫研究成果報告

＊ ********* ＊＊ ＊
＊ 計 畫 探討硫酸皮膚素表異構酶(DSE)在腦膠質細胞瘤的功能＊
＊ ： Investigating functions of dermatan sulfate ＊
＊ 名 稱 epimerase 1 in glioma progre ＊
＊ ********* ＊＊ ＊

執行計畫學生： 蔡侑桓
學生計畫編號： MOST 106-2813-C-040-019-B
研 究 期 間 ： 106 年 07 月 01 日至 107 年 02 月 28 日止，計 8 個月
指 導 教 授 ： 劉炯輝

處理方式： 本計畫涉及專利或其他智慧財產權,2 年後可公開查詢

執 行 單 位： 中山醫學大學醫學系解剖學科

中華民國 107 年 03 月 31 日

目錄

探討硫酸皮膚素表異構酶(DSE)在腦膠質細胞瘤的功能

Investigating functions of dermatan sulfate epimerase 1 in glioma progression

二、研究計畫內容（以 10 頁為限）：

英文摘要

Change of the extracellular matrix (ECM) in tumor microenvironment enhance glioma progression. Chondroitin sulfate exist in ECM and cell surface, it can be remodeled by dermatan sulfate epimerase to change its structure. The structure will change into chondroitin sulfate/dermatan sulfate (CS/DS) hybrid chain. Dermatan sulfate epimerase 1(DSE) was reported overexpressed in many types of cancer. However, the role DSE play in glioma is still unknown. We first search public database and run immunohistochemistry on tissue array. We found that DSE was up-regulating in glioma versus to normal brain tissue. High expression of DSE cause low survival rate in glioma and promote its tumor grade. We found DSE highly expressed in several glioma cell line and DSE directly produce DS chain in glioma cell. Knockdown of DSE suppressed proliferation, migration, and invasion of glioblastoma cells. In contrast, overexpression of DSE in GL261 cells enhanced these malignant phenotypes and in vivo tumor growth.

中文摘要

細胞外的胞外基質對於細胞的生理表現會起到調控作用，而對於腫瘤細胞來說這個調控就更起到關鍵作用，會影響到腫瘤是否會繼續惡化亦或是維持不變。而在胞外基質當中，含量最高且起到關鍵調控作用的非糖胺聚醣莫屬。糖胺聚醣是蛋白聚糖大分子中聚糖部分的總稱，而我們發現其中的硫酸皮膚素在腦瘤細胞中會大量表現，因而促成了本次研究的產生。透過免疫組織螢光染色法，我們在腦瘤病患的組織切片中發現了硫酸皮膚素的大量表現。更進一步的，我們透過基因轉殖法將實驗室培養的腦瘤細胞中的硫酸皮膚素的基因關掉。硫酸皮膚素基因被關掉的細胞，比起未關掉的細胞，細胞惡質性的表現降低了不少。這結果間接證明了硫酸皮膚素是造成惡性腦瘤的重要推手。

關鍵詞

Glioma; Glioblastoma; Dermatan sulfate; DS epimerase

（一） 前言

Brain cancer can classified into many types, the most common one is glioma (43% of all kind of brain tumor). Unfortunately, most of the glioma is malignant. Malignant glioma represents 1% of adult cancer worldwide, and is the third leading cause of cancer-related death in young age (Group less than 35 years)[1]. No matter treating by surgery or chemotherapy, the average survival is less than two years[2]. In this regard, finding the mechanism of cancer progression is important for creating new treatment to against this fatal disease.

Cancer cells or cancer-associated stromal cells produce extracellular matrix (ECM), and it influence angiogenesis, invasion and metastasis. That means aberrant expression of ECM protein in tumor microenvironment is a characteristic of all types of cancer[3]. Instead of collagen, central nerve system (CNS) stroma is filled with glycosaminoglycans (GAG) and proteoglycan [4]. GAGs are unbranched polysaccharide chains, such as chondroitin sulfate (CS) and dermatan sulfate (DS). Chondroitin /dermatan sulfate (CS/DS) proteoglycans appear both in matrix and cell surface, which could be important mediators in these malignant phenotypes. The main difference between DS and CS is the presence of iduronic acid (IdoA) in DS. The enzyme, DS epimerase 1(DSE), catalyzes GlcA into IdoA which is crucial for the expression of DS in human tissue. In the past researches, DSE was reported overexpressed in many types of cancer, such as lung cancer and squamous cell carcinoma. It was considered as a cancer-associated antigen. However, the role of DSE in glioma is still unknown.

（二） 研究目的

Introduction and significance

Various posttranslational modifications and glycosylation regulate different protein function and correlate with cancer progression and many other diseases[5]. DSE is a gene express in many types of cancer, such as melanomas, lung adenocarcinomas, and squamous cell carcinoma [6]. Therefore, we hypothesize it has a highly possibility concerned with the happen of glioma, it could regulate malignant behaviors of glioma cells.

Brain tumor is the second high cancer in children, only lower than blood cancer. Most malignant primary brain tumor develops from the cells that support the nerve cells of the brain called glial cells. A tumor of glial cells is a glioma[7]. Malignant glioma represent 1% of adult cancer worldwide, and is the third leading cause of cancer-related death in young age (Group less than 35 years) [8]. In Taiwan, malignant primary brain tumor diagnosed per year is about

600.Children age from 0~10 years old and elder age above 50 years old has a higher rate to get this cancer. The conventional treatment for glioblastoma is surgery, radiation and chemotherapy. Sometimes the anti-angiogenic agents were used to decrease the invasion and metastasis. However, the survival time of patient did not get long enough in our expectation since the efficacy of this method to high grade glioblastoma is limit [8]. Therefore, investigation of the precise molecular mechanisms underlying glioma progression is of great importance for developing new reagents to treat this aggressive cancer.

（三） 文獻探討

3.1 Glycosaminoglycans and proteoglycans

Glycosaminoglycans (GAGs) are unbranched polysaccharide chains which abundant in the extracellular matrix (ECM) of tumor as well as on the surface of cancer cells. GAGs exist as free chains or covalently link to core protein are known as proteoglycans (PG). There are many different types of these substance, keratan sulfate, hyaluronan, dermatan sulfate (DS), heparin, heparan sulfate (HS), and different isomeric forms of chondroitin sulfate (CS).The GAGs chain will link to some core protein, and it called proteoglycan. Both GAG as well as PG play important roles in regulating cell migration, morphogenesis , differentiation, cell growth[9].

Chondroitin sulfate (CS)/dermatan sulfate (DS) composed of alternating units of GalNAc and GlcA, or IdoA in the case of DS. The mainly different between CS and DS is the IdoA residue. In the past researches, the CS/DS family has been reported that they have effects on many physiological development, such as cell division and support structure in connective tissue. CS/DS target protein ligand including growth factors, selectins, and chemokines. It’s obviously that DS and CS are important in human body. The enzyme involved in the biosynthesis of DS is DSE [10].

3.2.1 DSE is up-regulated in glioblastoma

To see which tissues have the highest DSE expression, we search the gene in oncomine, a cancer microarray database. And just like what I thought, *DSE* express highly in brain tumor, especially in glioblastoma (Figure 1). The expression in glioblastoma is around two times higher than in normal brain tissue. The *p* value shows that they have significant difference in statistic (Figure 2). This result revealed that *DSE* could involve in carcinogenesis, and its overexpression in glioblastoma suggested that it could be a crucial regulator to mediate tissue from normal to tumor [11].

3.2.2 High expression of DSE is associated with poor survival in glioma.

We next analyzed the gene expression of *DSE* in REMBRANDT database, which is an open microarray dataset with more than 500 glioma samples. Interestedly, we found that glioma patients with high expression of *DSE* are associated with poor survival, comparing to patients with low *DSE* expression (Figure 3).

3.2.3 DSE is frequently up-regulated in grade IV glioblastoma cell lines.

To evaluate protein expression in human glioma cell lines, Western blot was carried out. Eight cell lines, GBM8401, GBM8901, A172, DBTRG-05MG, Ln18, Ln229, U118, and U251 were used as well as normal mouse brain tissue was taken as control. Results showed that DSE was up-regulated in grade IV glioblastoma cell lines, including A172, Ln18, U118, and U251 (Figure 4).

Collectively, data form online database indicated that *DSE* gene expression is frequently up-regulated in human glioma and is associated with worse survival. In addition, protein expression of DSE is up-regulated in certain human glioblastoma cell lines.

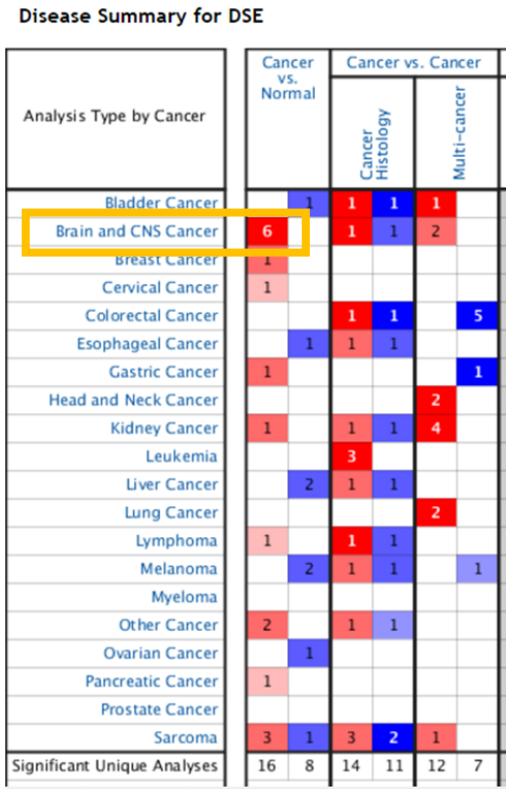

Figure 1. Expression of *DSE* in *ONCOMINE* cancer microarray database. Note that in brain and CNS cancer, *DSE* is up-regulated 6 datasets, which is much higher than other types of cancers. This data was from *ONCOMINE*: https://www.oncomine.org/resource/login.html.

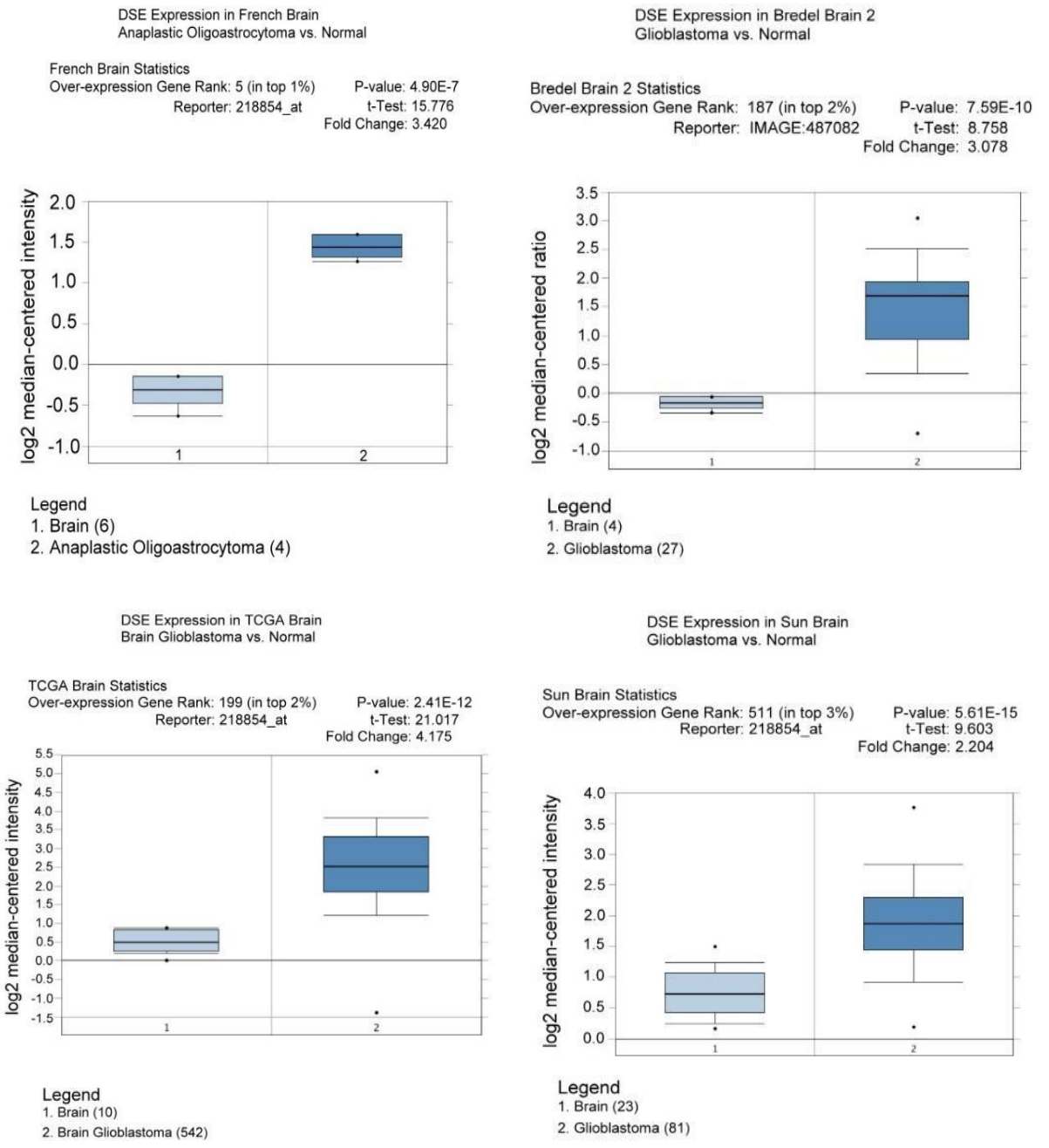

Figure 2. *DSE* is up-regulated in glioblastoma. Expression of *DSE* in glioblastoma tissue is significantly higher than that in normal brain tissue. Four independent datasets were shown. This data was from *ONCOMINE*.

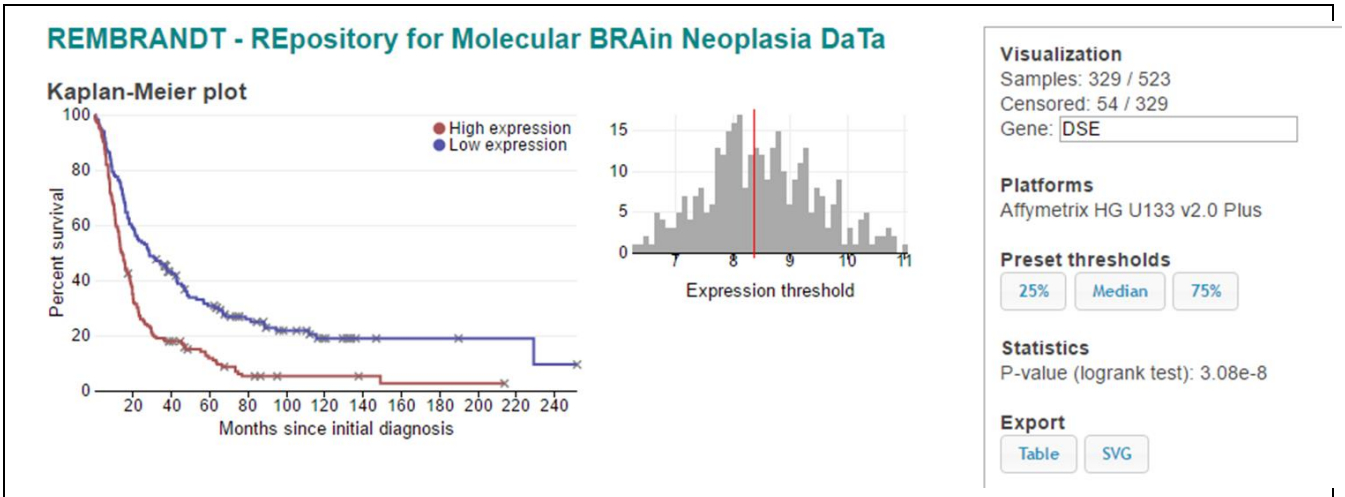

Figure 3. High expression of *DSE* is significantly associated with poor survival in glioma patients. This data was from REMBRANDT database, http://www.betastasis.com/glioma/rembrandt/.

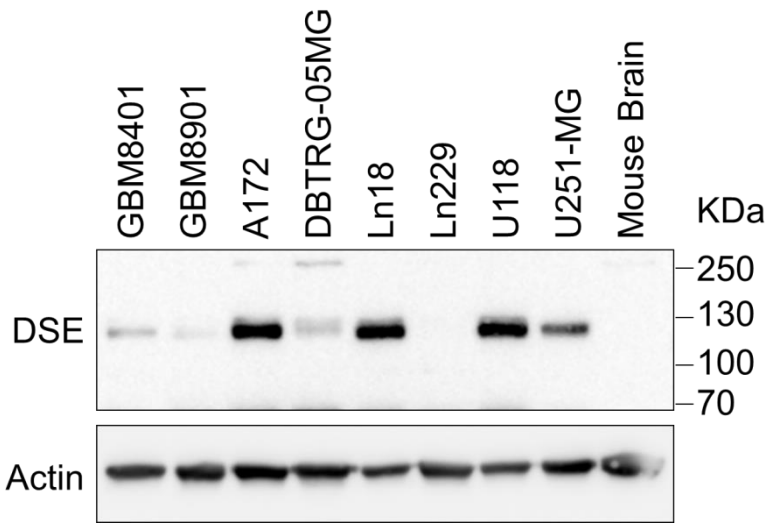

Figure 4. Protein expression levels of DSE in human glioblastoma cell lines and mouse brain tissue. Actin was used as internal control.

（四）研究方法

Immunohistochemistry

We use the immunohistochemistry stain to start the first experiment. We purchased paraffin-embedded normal human brain and glioblastoma tissue microarray which contain 5 normal brain tissue and 77 primary glioma tissue. We first incubated slide in 60 degree incubator, then used xylene and ethanol to dehydrate. After the step above, we followed staining protocol and used Thermo Scientific IHC kit and anti-DSE monoclonal antibody to finish the IHC stain.

Analysis of IHC

After IHC stain of these samples, the correlation between DSE expression and pathological characteristics was evaluated according to the information provided by supplier. We first used TissueFAXS Cytometry to take photo of the tissue array. Than used SAS-EG to run chi-square test to make data analysis.

Cell culture.

Malignant glioma cell lines. We buy GBM8401, GBM8901 and DBTRG-05MG from Bioresource Collection and Research Center in 2014(Hsinchu, TAIWAN). A172, Ln18, U118, GL261 and U251-MG were kindly provided from Dr. Wei KC(Chang Gung Memorial Hospital). The glioma cell lines were incubated in DMEM containing 10% FBS in 5%CO2 at 37°C.

Plasmid construction and stable clone selection

To produce stable DSE knockdown clones, DSE shRNA plasmids (purchased from National RNAi Core Facility) was first transfected into A172 and U118 cells using Lipofectamine 2000 (Invitrogen). Then the cultures was subsequently treated with 1000 ng/mL puromycin in the presence of 10% FBS in DMEM for 10 days to select stable DSE knockdown clones. Non-specific shRNA plasmids were used parallel as control cells. The DSE expression was checked by Western blotting.

Western blotting and DS chains blotting

Western blotting was carried out as reported previously. To check DS chains on proteoglycans, we used HRP conjugated-DS binding protein to blot DS chain and then visualized by ECL.

Analysis of cell proliferation assay

Cell growth was determined using the CCK8 assay. Cells was seeded at a density of 2×10^3 per well onto 96 well plates supplied with DMEM containing 10% FBS. After that, CCK8 solution (5 mg/ml) will be added to each well for 2 hour. Absorbance was measured at 495 nm using an ELISA-plated reader.

Analysis of cell invasion and migration in DSE-knockdowned transfectants.

24-well transwell inserts coating with Matrigel was used to analyze the cell invasion, and normal transwell without Matrigel was used to evaluate cell migration. Cell was starved in 0.1% serum for one day, and then seeded in the upper chamber with serum free DMEM. The lower chamber will filled with 10% FBS. After 16h incubation, chamber was removed; the membrane will fixed in methanol for 2 min and stained with crystal violet. We used Image J to count the number of cell migrating and invasion through the filter membrane and calculate the percentage of passed cells over the total incubated cells per culture.

Analysis of cell colony formation assay

We seeded cell in a density of 500 cells per well in a 6-well culture plate supplemented with DMEM containing 10% FBS. After 14 days, cell was fixed with methanol for 2 min and stained with crystal violet. Colony formed by each cell group was counted and measured.

Tumor growth mice models

8 weeks male mice were bought from National Laboratory Animal Center (Tainan, 21

Taiwan). 2.5 × 106 of GL261 mock transfectants and DSE transfectants cell were injected into right flank subcutaneously. After 15 days, we take out the tumor and used western blot to test the protein performance. All animal experiments in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Chung Shan Medical University Experimental Animal Center.

(五)結果與討論

DSE was up-regulated frequently in glioma where these events were associated with poor survival

Immunohistochemical analysis on tissue arrays containing 77 primary brain tumor tissues and 5 non-tumor brain to investigate the expression of DSE. Two-sided fisher's exact test was used to estimate survival rate of patients with glioma. Survival rate of high DSE expression was significantly lower than those with low DSE expression ($p<0.0024$) (Figure1)

DSE is a dominant dermatan sulfate synthase in glioma cells

We used western blot to check the protein expression levels of DSE in human glioblastoma cell lines and mouse brain tissue. Actin was used as internal control. And we found that DSE was highly expressed in both human and mouse glioblastoma cell. To simply measure the changes of DS chains in cell lysate, HRP-conjugated DS binding protein was used on Western blots. And the result prove that DSE directly catalyzes the formation of DS chains on several proteoglycans in glioma cells. (Figure 2)

DSE regulates malignant phenotypes in glioma cells

To investigate effects of DSE on malignant phenotypes, we used knockdown clone to test the proliferation, migration and invasion ability. Our data demonstrated that knockdown of DSE significantly suppressed cell proliferation, migration and invasion ability in U118 cells .These

data showed that DSE can modulate cell proliferation, migration, invasion, and in vivo tumor growth of glioma cells. (Figure3)

Discussion

Data established that up-regulated DSE expression cause malignant phenotype in glioblastoma.

DSE affect the formation of DS chain, then CD/DS hybrid chain enhanced the cancer level of brain cancer. The later cancer level cause malignant phenotype of the cancer. The worse phenotype showed in many way such as strong invasion and migration level of the cancer cell. These bad behaviors may cause bad prognosis after surgery or chemical therapy. It's not a good news for patient. However, we have found the prominent substance, DSE, which lead this disease become worse. In the future, therapeutic targeting DSE might extend novel strategies to decrease death rate and relapse of this disease.

（六）參考文獻

1. Ostrom, Q.T., et al., *CBTRUS Statistical Report: Primary brain and other central nervous system tumors diagnosed in the United States in 2010-2014.* Neuro Oncol, 2017. **19**(suppl_5): p. v1-v88.
2. Omuro, A. and L.M. DeAngelis, *Glioblastoma and other malignant gliomas: a clinical review.* JAMA, 2013. **310**(17): p. 1842-50.
3. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
4. Quail, D.F. and J.A. Joyce, *The Microenvironmental Landscape of Brain Tumors.* Cancer Cell, 2017. **31**(3): p. 326-341.
5. Ohtsubo, K. and J.D. Marth, *Glycosylation in cellular mechanisms of health and disease.* Cell, 2006. **126**(5): p. 855-67.
6. Nakao, M., et al., *Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL.* J Immunol, 2000. **164**(5): p. 2565-74.
7. TCOG 顱內腫瘤研究委員會, *腦瘤之診斷與治療共識.* 1 ed. 2004-10, 台灣: 國家衛生研究院. 38.
8. Fang, K.M., et al., *Induced interleukin-33 expression enhances the tumorigenic activity of rat glioma cells.* Neuro Oncol, 2014. **16**(4): p. 552-66.
9. (NY), C.S.H., *Essentials of Glycobiology*. 2nd edition ed. 2009, Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.
10. Maccarana, M., et al., *Biosynthesis of dermatan sulfate: chondroitin-glucuronate C5-epimerase is identical to SART2.* J Biol Chem, 2006. **281**(17): p. 11560-8.
11. Thelin, M.A., et al., *Dermatan sulfate is involved in the tumorigenic properties of esophagus squamous cell carcinoma.* Cancer Res, 2012. **72**(8): p. 1943-52.

Figure

Figure 1. DSE is frequently up-regulated in human glioma.

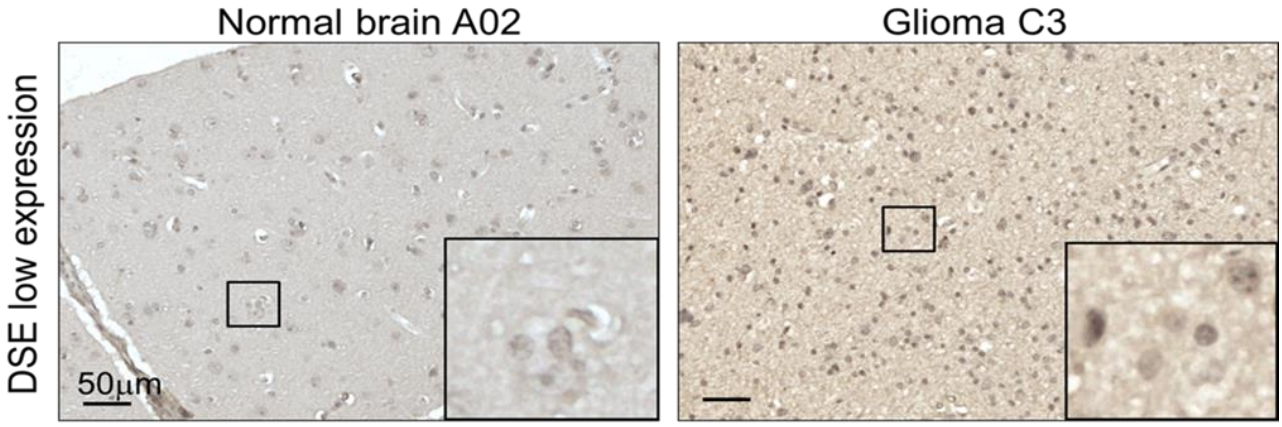

Table 1. Correlation of DSE expression with clinicopathlogical features of glioma tissue array.

Factor		DSE expression		
		Low	High	*P* value (Two-sided Fisher's exact test)
Tissue types	Non-tumor	5	0	0.0024*
	Tumor	21	56	
Sex[#]	Male	8	27	0.785
	Female	9	25	
Age[#]	< 55 years	10	20	0.167
	≥ 55 years	7	32	
Tumor stage	Grade I – III[$]	14	19	0.019*
	Grade IV (GBM)	7	37	

*$P < 0.05$ was considered as statistically significant.
[#]Eight patients' sex and age were not provided.
[$]Astrocytoma and Oligodendroglioma.

Figure 2. DSE regulates dermatan sulfate formation in glioma cells.

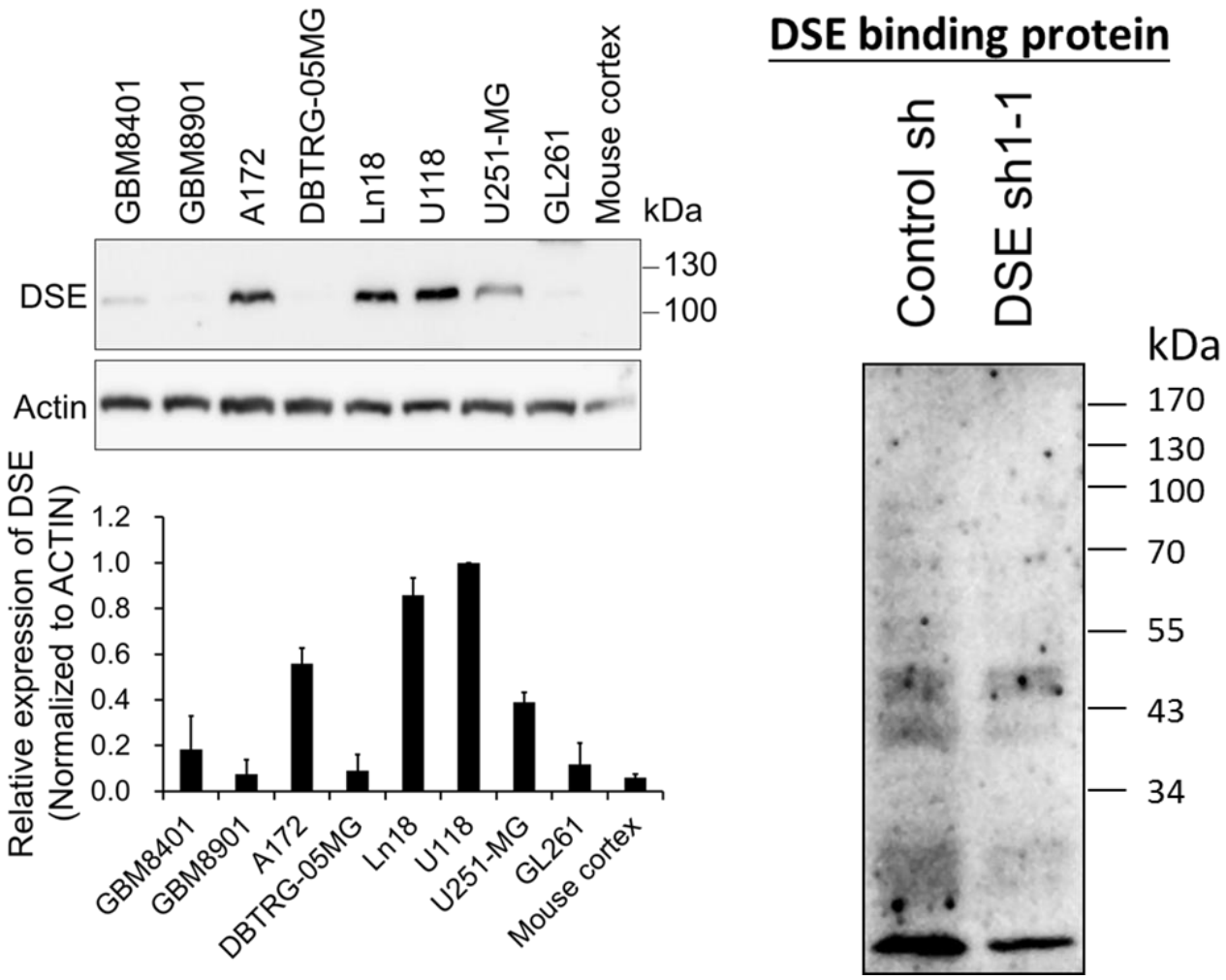

Figure 3. Expression of DSE regulates malignant phenotypes in glioma cells.