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<tr><td>計　畫
名　稱</td><td>：</td><td>硫酸軟骨素生成酶在人類膠質細胞瘤的表現以及功能之
研究</td></tr>
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Abstract

Glioma is one of the most aggressive cancer among all the primary brain tumors. It involves in the central nerve system, and can be divided into several subtypes depending on the appearance and histopathological features. World Health Organization classifies it into four grades based on their appearance, and glioblastoma (GBM) is the most malignant one. These tumors tend to grow and infiltrate into the normal brain tissue.

Malignant glioma is the third-leading cause of cancer death in less than 35 years population worldwide and it is almost incurable currently. The average rate of glioblastoma survival is less than 2 years, even in patients who have received standard surgical resection followed by radiation and chemotherapy, or enrollment in a clinical trial. Currently, patients with glioma still cannot receive extremely effective treatment though.

Glycosaminoglycans (GAG) are long unbranched polysaccharides consisting of a repeating disaccharide unit and they exist in the extracellular matrix of central nerve system. GAG can be classified into several types, especially chondroitin sulfate (CS), plays important roles in CNS development, cell growth, cell differentiation, cell migration, and cell-cell interaction, which attribute to carcinoma proliferation. It is well known that CS chains are major component of glial scar which prevent nerve regeneration. Research shows that CS and CS proteoglycans (CSPG) are abnormally accumulated in human glioma, but the underlying mechanisms remain unexplored.

The formation of CS glycan chains is catalyzed by CS synthases, including CSGALNACT1, 2 and CHSY1, 2, 3. But which enzymes governing the accumulation of CS chains in CNS, the biological functions of CS chains in glioma progression, and the relationship between glioma cell phenotypes and the aberrant expression of CS synthase family still remain largely unknown.

This study suggests that the expression of CHSY1 is up-regulated in glioma. And it also correlates with the biological features and phenotypes of glioma. CHSY1 can modulate glioma malignancy, histology grade, clinicopathlogical features, and cell proliferation. Consequently, CS synthases play great roles on modifying aberrant accumulated GAG, thus regulate glioma cell properties.

Keywords

Glioma; Glioblastoma; Chondroitin sulfate; Chondroitin sulfate synthase; CHSY1

Abbreviations

CNS: central nerve system; GBM: glioblastoma; WHO: World Health Organization; ECM: extracellular matrix; PG: proteoglycans; GAG: glycosaminoglycan; CS: chondroitin sulfate; DS: dermatan sulfate, KS: keratan sulfate, HS: heparan sulfate; CSPG: chondroitin sulfate proteoglycan

1. Introduction

Glioma is a type of tumor which involves in the central nerve system (CNS), and it originates from glial cells around nerve cells. It is the most common type of primary brain tumors. Gliomas can be classified into several types of cancers, like astrocytomas, oligodendrogliomas, ependymomas and glioblastoma (GBM), depending on the location, rate of growth, histopathological features and clinical presentation. It is also divided into four grades based on their appearance by World Health Organization (WHO). Grade I is the lowest grade, while Grade IV is the highest one, which can be GBM or malignant astrocytoma/oligodendroglioma. GBM is the most malignant and most aggressive type of glioma, it is defined by the hallmark features of uncontrolled cellular proliferation, diffuse infiltration, propensity for necrosis, robust angiogenesis, intense resistance to apoptosis, and rampant genomic instability (Frank et al, 2007). Many research show that tumor cells influence brain functions and can be life-threatening. These tumors tend to grow and infiltrate into the normal brain tissue. Among the most aggressive human .ancers, malignant glioma is the third-leading cause of cancer death in less than 35 years population worldwide. (Ostrom et al, 2017) In Taiwan, 600 new cases of malignant primary brain tumor diagnosed per year. Currently, malignant gliomas are almost incurable. The average rate of glioblastoma survival is less than 2 years, even in patients who have received standard surgical resection followed by radiation and chemotherapy, or enrollment in a clinical trial. The high mortality of this disease is mainly attributable to the limited treatment options and the almost inevitable recurrence after surgical care (Omuro & DeAngelis, 2013; Stupp et al, 2005). Therefore, elucidation of the precise molecular mechanisms underlying glioma progression is of great importance for the development of new reagents to treat this fatal disease.

Remodeling of the extracellular matrix (ECM) in the tumor microenvironment is a hallmark of cancer (Hanahan & Weinberg, 2011). Different from other organs, constituents of ECM in CNS stroma is abundant with glycosaminoglycans (GAG) and proteoglycans (PG), instead of regular ECM proteins such as collagens or laminins (Quail & Joyce, 2017). GAG are long unbranched polysaccharides consisting of a repeating disaccharide unit (Adesuyi et al, 2012). They are presented as free chains or covalently link to core protein known as PG which are major located at extracellular, cell surfaces, and in the cytoplasm (Liu et al, 2017; Kristian,2015). GAG can be classified into five types, including chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) ,and heparan sulfate (HS). GAG, especially CS, plays crucial roles in CNS development, cell growth, cell differentiation, cell migration, and cell-cell interaction, which attribute to carcinoma proliferation (Akita et al, 2008; Sugahara et al,

2003; Yamada & Sugahara, 2008). It is well known that CS chains are major component of glial scar which prevent nerve regeneration. Research show that CS and CS proteoglycans (CSPG) are abnormally accumulated in human glioma, but the underlying mechanisms remain unexplored.

The formation of CS glycan chains is catalyzed by CS synthases, including CSGALNACT1, 2 and CHSY1, 2, 3. In human, the N-acetylgalactosamine linking to a tetrasaccharide structure is initiated by CSGALNACT1, CSGALNACT2 transferases. Then, CS chains were polymerized by CHSY1, CHSY2 (CHPF), and CHSY3 (Liu et al, 2017). The expression of CS synthases in glioma is different from normal brain tissue and have influence on glioma malignancy. Using Chondroitinase ABC or CSPG inhibitor to eliminate CS chains deposit in the lesioned dorsal columns promoted functional recovery of spinal injuries (Bradbury et al, 2002; Keough et al, 2016). In glioma, previous studies showed that several CSPG, such as neurocan and brevican, significantly increased in tumor ECM and associated with malignant behavior of cancer cells (Viapiano M.S., 2009; Wade et al, 2013). Degradation of CS in glioma by oncolytic viral expressing bacterial Chondroitinase ABC enhanced virus spread and anti-tumor efficacy of these virus (Dmitrieva et al, 2011). Moreover, a recent study found that cleaving CS chains enhanced temozolomide availability and sensitivity to glioma cells (Jaime-Ramirez et al, 2017). Although these studies highlight the importance and therapeutic targeting potential of CS chains in CNS, which enzymes governing the accumulation of CS chains in CNS, the biological functions of CS chains in glioma progression, and the relationship between glioma cell phenotypes and the aberrant expression of CS synthase family still remain largely unknown.

2. Materials and methods

Cell culture

Glioma cell lines, A172 cells were kindly provided by Dr. Wei KC (Chang Gung Memorial Hospital). Cells were cultured in DMEM containing 10% FBS in at 37°C.

Reagents and antibodies

CCK8 reagent and antibody against CHSY1 were purchased from Sigma-Aldrich. ON-TARGETplus SMARTpool siRNA against human *CHSY1* was purchase from Dharmacon. Full length CHSY1-pcDNA3.1 plasmid was purchased from GeneScript. Two pLKO.1/CHSY1-shRNA plasmids and nontargeting pLKO.1 plasmids were purchased from National RNAi Core Facility (Academia Sinica, Taipei, Taiwan).

Transfection

For transient knockdown of CHSY1, cells were transfected with 20 nmol of siRNA using Lipofectamine RNAiMAX (Invitrogen) for 48 hours.

Tissue array and immunohistochemistry

UltraVision Quanto Detection System (Thermo Fisher Scientific Inc.) was used for immunohistochemistry. Paraffin-embedded human Glioma tissue microarrays were purchased from Shanghai Outdo Biotech and Pantomics, Inc. Arrays were incubated with anti-CHSY1 antibody (1:100) in 5% bovine serum albumin/PBS 16 hours at 4°C. The specific immunostaining was visualized with 3,3-diaminobenzidine and counterstained with hematoxylin (Sigma). Images were obtained by TissueFAX Plus Cytometer.

Cell viability

Cell viability was analyzed by CCK8 assay at 0, 24, 48, and 72 hours following manufacture's protocol. Cells (2×10^3) were seeded into 96-well plates with culture medium.

Cell invasion and migration assay

Matrigel (BD Biosciences) coated porous filters were used to evaluate cell invasion, and transwell inserts with uncoated porous filters (pore size 8 μm) were used to estimate cell migration. 2×10^4 cells in serum-free DMEM were seeded into inserts, DMEM containing 10% FBS was added in lower part of the inserts for 16 hours incubation. Independent experiments were repeated for at least three times. Average number of cells per microscopic field was shown.

Statistical analysis

All data analysis was performed by GraphPad Prism 6. Mann–Whitney U test was used for analysis the relationship of immunohistochemistry and glioma tissue arrays. Two-sided Fisher exact test was used for comparisons between clinicopathologic features of glioma tissue array and CHSY1 expression. Student t test was used for statistical analyses. $P < 0.05$ was considered statistically significant.

3. Results

The expression of CHSY1 is up-regulated in human glioma and associates with poor survival and high malignancy of glioma subtypes.

CS synthases expression in gliomas were different from normal brain tissue. The synthases could affect the structure and microenvironment of CNS greatly. To find out the relationship between the expression of CS synthases and the characterization of gliomas, clinical and functional genomics data from clinical

trials involving patients suffering from gliomas would be necessarily needed. The Kaplan-Meier curve according to the REMBRANDT database indicated that the survival rate for patients with glioma. Among all the CS synthases, CHSY1 modified gliomas malignancy the most. Also, it showed that the survival of patients with gliomas in high CHSY1 expression was markedly worse than those with low CHSY1 expression. (Log-rank test, P = 5.90E-the 7; Fig. 1A) It came to the fact that the expression of CHSY1 was up-regulated in glioma and correlated with poor survival. We mentioned if CS synthases had also relatively association with the level of malignancy in glioma subtypes. Glioma was majorly divided into astrocytomas, oligodendrogliomas, and glioblastoma depending on its location, rate of growth. Among all the subtypes of gliomas, glioblastoma was the most aggressive and malignant one. As mentioned previously, the same CS synthases, CHSY1, was used as control group and standard. To compared CHSY1 gene expression in glioma subtypes and normal brain tissue from REMBRANDT database, CHSY1 expression in patients with glioblastoma was the highest, while in those with normal brain tissue was the lowest. (****P<0.0001; Fig. 1B) Together, the expression of CHSY1 was up-regulated in glioma and correlated with poor survival and high malignancy of glioma subtypes.

High histology grades of human glioma is positive correlative with the expression of CHSY1.

The human gliomas were divided into four grades based on their appearance, histopathological features and clinical presentation by WHO. While the most aggressive one, like GBM, was classified into Grade IV, and Grade I was the least one. The immunohistochemical analysis on tissue arrays contained 85 cases of patients with glioma and 5 cases of those with normal brain tissue. The immunohistochemistry was conducted to show the expression of CHSY1 in the cytoplasm and paranuclear of brain cells. (Fig. 2A) The intensity of staining was then scored with the percentage of CHSY1-positive cells in each sample. (0, < 5%; +1, 5%–20%; +2, 20%–50%; +3, > 50%) The data were classified into four grades and normal tissue according to the information by supplier. Combined with the immunohistochemistry and clinicopathlogical data, it showed that high intensity of CHSY1 expression took up the largest proportion in Grade IV.(Mann–Whitney U test, P=0.0003; P<0.001; P=0.0147), and low intensity of CHSY1 expressed in normal brain tissue (Fig. 2B) From these data, high histology grades of human glioma were suggested to have positive correlation with the expression of CHSY1.

From the clinicopathlogical features of glioma tissue array, patients with high expression of CHSY1 are more likely to have tumor tissue (tissue types) and high grade of glioma (tumor stage).

Based on the glioma tissue array mentioned above, the correlation of CHSY1 expression with clinicopathlogical features were analyzed . The CHSY1 expression was classified into two groups (high

and low). Factors were provided, including tissue types, sex, age and tumor stage. According to the analysis, patients with glioma tumor-tissue-type tended to have high CHSY1 expression which was 66% (56/85), while only 0% (0/5) in non-tumor-tissue expressed high levels of CHSY1.(Two-sided Fisher's exact test, P=0.0063) From the tumor stage side, our data revealed that CHSY1 highly expressed in 85% (35/41) of Grade IV(GBM), while only 48% (21/44) in Grade I-III revealed high expression of CHSY1 (Two-sided Fisher's exact test, P=0.0003) As a result, we could infer that increased CHSY1 expression was correlated with tumor-tissue type and advanced tumor stage of glioma tumors.(Table 1.)

Knockdown of CHSY1 decreases signaling and binding in glioma cell line. Thus, the presence of CHSY1 can help enhance cellular proliferation in glioma.

Structural domains in chondroitin sulfate could be identified by anti-chondroitin sulfate monoclonal antibodies (CS-56) (J. Michael et al, 1993). A172 were glioma cells we used. Flow cytometry with anti-CS56 antibody was used to detect signaling. Control-siRNA (Ctr-si) and CHSY1-siRNA (CHSY1-si) respectively modulated the chondroitin sulfate in glioma cell which could be identified by CS-56. It came to the result that knockdown of CHSY1 could decrease CS-56 signaling. (Fig. 3A) Geometric mean fluorescence intensity index (Geo-MFI) method was used to measure CHSY1 expression in patients with glioma. Non-specific mouse IgM was used as iso-type control. Knockdown of CHSY1 decreased Geo-MFI. (Fig. 3B) Knockdown of CHSY1 decreased signaling and binding in glioma cell line. Thus, the presence of CHSY1 could help enhance the quantity of chondroitin sulfate in glioma.

Knockdown of CHSY1 decreases cell viability, migration and invasion, related to less malignant phenotypes in glioma cell line.

Due to the up-regulation of CHSY1 expression to malignancy and histology grades in gliomas, cell viability, invasion and migration assay were conducted to see whether CHSY1 correlated with malignancy phenotypes in glioma cell line. Cell viability was determined using a CCK-8 assay. A172 were glioma cells we used. It showed that knockdown of CHSY1 decreased cell viability. (T test, P < 0.01; Fig. 4A) In addition, Control-siRNA (Ctr si) and CHSY1-siRNA (CHSY1-si) cells were subjected to transwell migration assay and Matrigel invasion assay. Knockdown of CHSY1 also decreased cell migration and invasion. (T test, P < 0.001; Fig. 4B and 4C) To sum up, these data suggested that expression of CHSY1 could modulate phenotypes in glioma cell line. Moreover, knockdown of CHSY1 decreased cell viability, migration and invasion, related to less malignant phenotypes in glioma cell line.

4. **Discussion**

The results obtained in this study suggest that the expression of CHSY1 is up-regulated in glioma. And it also correlates with poor survival and high malignancy of glioma subtypes. Besides, high histology grades of human glioma are positive correlative with the expression of CHSY1. From the clinicopathlogical features of glioma tissue array, patients with high expression of CHSY1 are more likely to have tumor tissue and high grade of glioma. We then demonstrate that knockdown of CHSY1 decreased signaling and binding in glioma cell line. Thus, the presence of CHSY1 can help enhance cellular proliferation in glioma. And knockdown of CHSY1 decreases cell viability, migration and invasion, related to less malignant phenotypes in glioma cell line. Together we obtain that CHSY1 is one of the critical enzymes which dominants glioma cell malignancy.

Many previous researches have demonstrated that glioma tumor cell can be regulated by CHSY1. One of the studies shows that NOTCH signaling pathway can be modulated by CHSY1 levels. CHSY1 expression is significantly up-regulated with relative transcript abundance of JAG1, a NOTCH ligand, and HES1, an immediate target of NOTCH in comparison to non-tumor cells. CHSY1 as well as other CS synthases involves with intracellular signal transduction. Currently, precise molecular mechanisms are the parts we should pay attention to. Finding out which pathway that worsens tumor cell properties, we need to look into CS synthases more by Western blotting and qPCR to confirm the downstream signaling.

Also, CHSY1 can influence tumor progression in other types of tumor. It regulates NFκB and caspase-3/7 signaling in RKO cells, which promotes cell proliferation and apoptosis in colorectal cancer (Zeng et al, 1993). Another study shows that silencing the expression of CHSY1 in human fibroblast cells can lead to reduced expression of caspase 1, which is a kind of protease that regulates cell proliferation and migration (George et al, 1993). These findings point out that CHSY1 is one of significant enzyme that modulate carcinoma proliferation, and it means CHSY1 is the important therapeutic targets for many kinds of tumor. According to the studies mentioned above, gaining more novel insights into the pathogenesis of the disease may help finding out the way that reduces tumor cell proliferation and improves malignant phenotypes of tumors.

In conclusion, this study proves that CHSY1 can regulate the phenotypes of human glioma. Though other mechanisms that influence tumor cells are still unknown, this study shows that chondroitin sulfate synthases have direct effects on glioma progression. It sheds light on the biological function of aberrant CS expression in human glioma. There's no effective treatment for patients with glioma, so targeting on

CHSY1 may become the major point of new therapy not only for glioma but also for other critical cancers.

5. Acknowledgements

6. Reference

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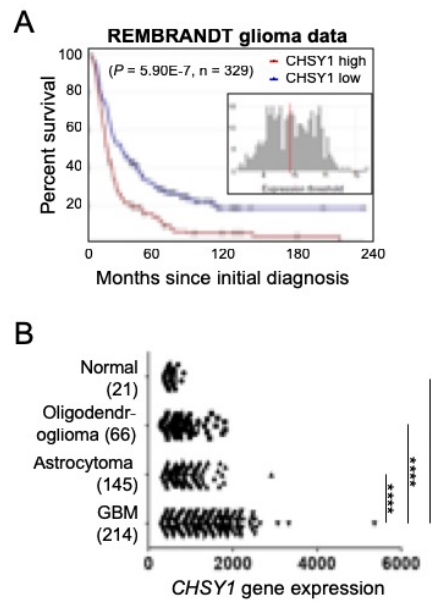
Figure 1

Fig. 1. The relationship between CHSY1 expression with the survival of glioma patients and human glioma subtypes.

(A) Kaplan-Meier curve of overall survival for patients with glioma. The analyses are according to the REMBRANDT database. Log-rank test, P = 5.90E-7. (B) Comparison of CHSY1 gene expression in glioma subtypes and normal brain tissue. (****P<0.0001) These data are from the REMBRANDT database.
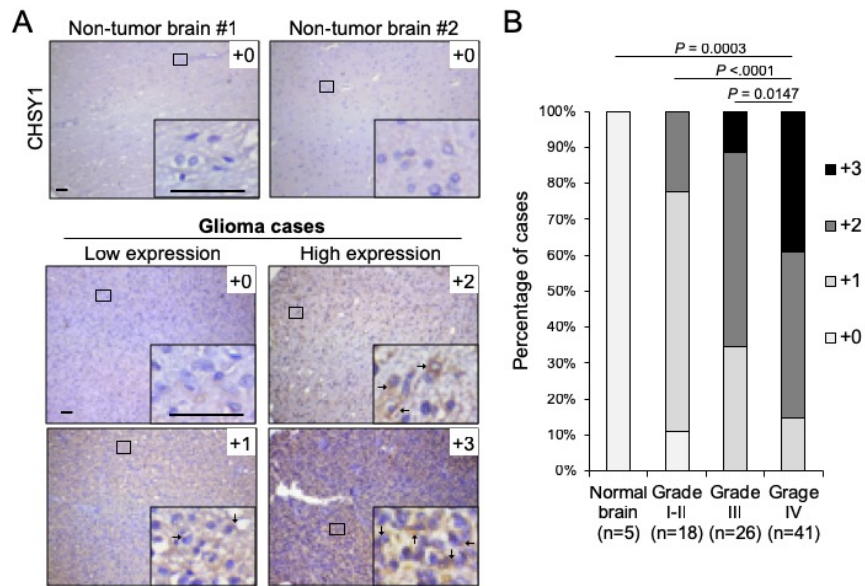
Figure 2

Fig. 2. CHSY1 expression in association with human glioma histology grade.

(A) Immunohistochemistry of CHSY1 in non-tumor brain tissue and primary glioma tissue arrays contains 90 cases. All sections were counterstained with hematoxylin. Representative images of CHSY1 low expression cases (middle) and CHSY1 high expression cases (right) were shown. Amplified images were shown at the bottom right of each image. Scale bars, 100 μm. (B) The intensity of staining was scored according to the percentage of CHSY1-positive cells in each sample, based on immunohistochemistry of CHSY1 in non-tumor brain tissue and primary glioma tissue arrays contains 90 cases. (0, < 5%; +1, 5%–20%; +2, 20%–50%; +3, > 50%) The data were classified into four grades and normal tissue with clinical information by supplier. The ratio of scores in each grade are shown. Mann–Whitney U test was used, and the P values were shown on the top.

Factor		CHSY1 expression		P value (Two-sided Fisher's exact test)
		Low	High	
Tissue types	Non-tumor	5	0	0.0063*
	Tumor	29	56	
Sex[#]	Male	8	32	0.0079*
	Female	18	18	
Age[#]	< 55 years	13	17	0.2192
	≥ 55 years	13	33	
Tumor stage	Grade I – III[$]	23	21	0.0003*
	Grade IV (GBM)	6	35	

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

Table 1. Correlation of CHSY1 expression with clinicopathlogical features of glioma tissue array.
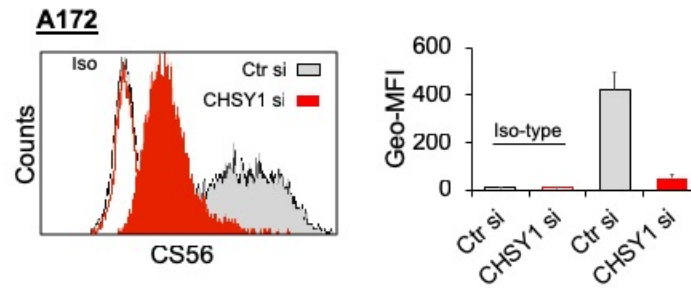
Figure 3

Fig. 3. CHSY1 regulates signaling and binding in glioma cells.

(A) Signaling modulated by Control-siRNA (Ctr si) and CHSY1-siRNA (CHSY1-si) in A172 cell. Flow cytometry with anti-CS56 antibody was used. (B) Geometric mean fluorescence intensity index (Geo-MFI) method was used to measure CHSY1 expression in patients with glioma. Non-specific mouse IgM was used as iso-type control.
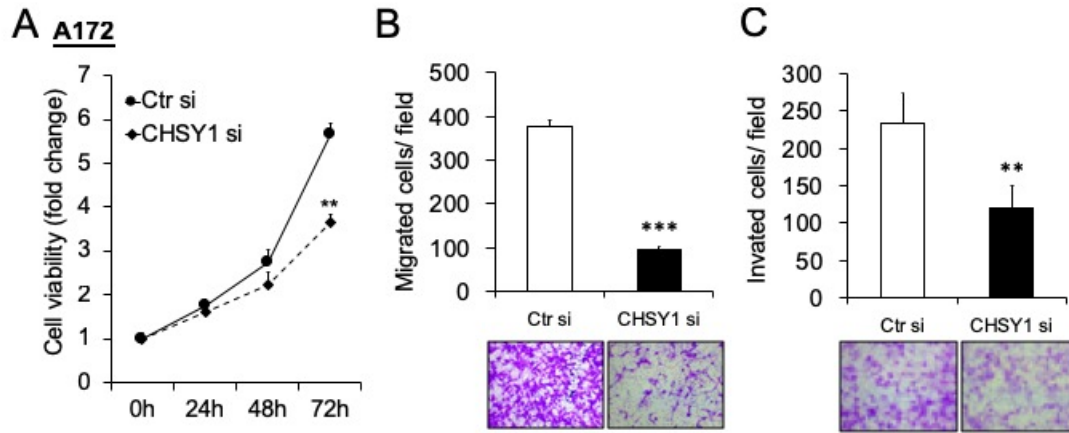
Figure 4

Fig. 4. CHSY1 modulates malignant phenotype of glioma cells.

(A) Knockdown of CHSY1 in A172 cell to measure the cell viability. Cell viability was determined using a CCK-8 assay. (B & C) Control-siRNA (Ctr si) and CHSY1-siRNA (CHSY1-si) cells were subjected to transwell migration assay (B) and Matrigel invasion assay (C). Representative images are shown at the bottom. *P < 0.05; **P < 0.01; ***P < 0.001.