Establishment and characteristics of GWH04, a new primary human glioblastoma cell line

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Abstract

Glioblastoma (GBM) is a common and fatal disease of the central nervous system. It has a worldwide occurrence and affects people from diverse backgrounds. GBM cell lines are fundamental tools used in GBM research. The establishment of novel continuous GBM cell lines with clear genetic backgrounds could facilitate the exploration of molecular mechanisms and the screening and evaluation of antitumor drugs in GBM studies. Here, we established a novel primary glioblastoma cell line, named GWH04, from a patient with glioblastoma, and examined its short-tandem repeat (STR) genotype and various tumor parameters. The STR information of GWH04 was identical to that of the original primary tumor tissue. Compared with existing cell lines, GWH04 had a similar in vitro proliferation rate as U87 cell line, but a faster rate than GL15 cell line, and a substantial soft agar clone formation capacity. GWH04 showed subcutaneous and intracranial tumorigenic capacity, with an aneuploid karyotype and median 84 chromosomes. Gene sequencing further revealed the mutational status of genes *TP53*, *PTEN*, *PDGFRA*, *ERBB2*, *BRCA1*, *NF1*, and *MLH1* and the promoter region of *TERT* (C228T) in this cell line. Altogether, these results indicate that GWH04 can facilitate human GBM studies both in vitro and in vivo.

Introduction

Malignant gliomas are the leading cause of central nervous system tumor-related deaths and affect more than 50% of glioma patients. Unfortunately, patients who are affected by glioblastoma multiforme (GBM) have a poor prognosis, with a mean life expectancy of less than 2 years, as existing therapies lack efficiency^{1,2}. As the overall survival of GBM patients has not improved much in the last 20 years, the current gold standard for GBM treatment is only palliative, including surgery, adjuvant radiotherapy, and temozolomide chemotherapy. One of the major hurdles in the development of novel treatment regimens for GBM is the challenge of translating scientific knowledge from bench to bedside, mainly owing to the fact that most research models only poorly replicate the tumor behavior and, consequently, many drugs that perform well in glioma models ultimately fail in clinical trials.

To date, malignant glioma cultures, especially publicly available cell lines, such as those obtained from the American Type Culture Collection (ATCC), represent a useful experimental tool for studies on glioma formation and progression, discovery of new antitumor strategies, and for the identification of molecular markers in response to conventional chemotherapies and targeted agents showing clinical utility. In a search for articles associated with original glioma cell cultures, we found that cell lines bearing the prefix “U,” such as U251, U373, and U87, were established in the Uppsala University, Sweden^{3,4}, and cell lines bearing the prefix “LN,” such as LN-18, LN-229, LN-464, were established at the Neurosurgical Service of the University Hospital (CHUV) in Lausanne, Switzerland^{5,6}. Searching for articles associated with the tumorigenicity of glioma cell lines, we found that U87, U251, U373, LN-18, LN-229, LN-464 are tumorigenic, whereas A172 and HS683 are nontumorigenic^{7,8}. Moreover, more than 1370 and 880 entries in PubMed in the past 3 years were of articles associated with U87 and U251, respectively, whereas only

210 and 17 entries were associated with A172 and HS683. This confirmed that the tumorigenicity of these GBM cell lines is firmly associated with their popularity in cancer research studies.

Although the abovementioned cell lines have been established and widely used for more than 40 years, they have several drawbacks that need to be considered. For example, in vitro subculture poses a selection pressure on cell lines, which may result in genetic drift over time. In addition, long-term cultures represent a risk of cross-contamination with other cell lines. The most commonly used cell line, U87, from the ATCC, which has been passaged worldwide for more than 50 years, was originally established in an Uppsala laboratory in 1966, yet one article in 2016 has shown that its origin is different from that of U87 from Uppsala or any other laboratory-established glioma cell line^{7,8}. Furthermore, U373 and U251 were first established as two different cell lines, but recent short-tandem repeat (STR) identification confirmed that they have the same origin (https://web.expasy.org/cellosaurus/CVCL_0021; https://web.expasy.org/cellosaurus/CVCL_2219). Thus, many journals require cell line STR authentication and primary cell culture to ensure the reliability of the studies. Therefore, new cell lines are urgently required to replace those old, long-term-use cell lines.

During the past years, we have routinely established several adherent primary cultures from freshly resected tissues of patients with different grades gliomas, and established one permanent and tumorigenic cell line, named GWH04, from a 72-year-old female patient with GBM without additional genetic modification. This cell line is suitable for the common culture medium and has been sub-cultured more than 70 times, and cryopreserved at different early passage stages. In this report, we describe the establishment of this primary GBM cell line and present its biological characteristics including proliferation rate, karyotype analysis, IC50 of different chemotherapy drugs, and glioma-associated gene test, as well as the pathological and histological characteristics of the original tumor from the patient, and intracranial xenografts in nude mice related to this cell line. This GWH04 cell line (first named as Fu) was deposited at the Bio-research Innovation Center (BRICS), Suzhou, China (http://www.brics.ac.cn/cell/template/cell/cell_detail.html?id=3825), and also preserved in the China Center for Type Culture Collection (CCTCC, NO.C202163), Wuhan, China. This cell line will contribute to the diversity of GBM cell lines and will be a useful tool for studies of human glioblastoma both in vitro and in vivo for all researchers.

Results

Primary culture and identification of GWH04 cells

In this study, we comprehensively investigated 10 cases of primary cell cultures from different GBM tumor tissues, among which, GWH04 cells could well circumvent replicative senescence and acquire the ability to sustain unlimited proliferation in this culture medium. The information of the patient from whom GWH04 cells were derived and the culture details are presented below.

CT and MRI scans of the brain tumor, and H&E staining of the paraffin-embedded tumor tissue of the patient are shown in Fig. 1. The histopathological diagnosis was GBM, based on the H&E staining, which showed numerous nuclear atypia, mitoses, and microvascular proliferation, and different IHC staining items (Supplementary Fig. 1).

After surgery, fresh tumor tissues were treated as described in the “Material and Methods” section. Primary cells were harvested and cultured in DMEM with 10% FBS, similar to the protocols used for U251 and U87 cultures. GWH04 began to grow significantly faster in the 7th generation, and cell morphology was intended to be stable, as shown in passage 10 (P10) and passage 50 (P50), compared with passage 1 (P1) and 2 (P2) (Fig. 2a). Immunofluorescence staining was performed in the 10th generation, showing that GWH04 cells were positive for GFAP and Nestin expression (Fig. 2b).

A previous STR study of 482 different human tumor cell lines used in China showed that up to 96 cell lines were misidentified⁹, and Nature research journals, AACR publications, and certain other scientific publishers currently require cell identification by DNA analysis of the samples and cell lines used. Herein, STR authentication of 20 locations (covering the nine loci demanded by the ATCC) from the tumor tissue of the patient and GWH04 cells was tested and a complete match was observed (Fig. 2c). Moreover, the STR profile differed from that of all cell lines available in different cell banks (<http://cellresource.cn/str/default.aspx>).

Genetic diagnoses of GWH04 cells

According to the 2016 World Health Organization Classification of Tumors of the Central Nervous System¹⁰, genetic diagnosis is recommended to identify the mutational status of *IDH1/2*, *TERT*

promoter, and *BRAF*, as well as 1p/19q co-deletion and *MGMT* promoter methylation, which can help predict prognosis and decide direct treatment options. These are the most common mutational combinations in GBMs, and specific combinations of these mutations often indicate a poor prognosis in clinical patients and call for a more aggressive treatment. In this study, we harvested the GWH04 cells (P7) and extracted the DNA for detection. There was no 1p/19q co-deletion or *IDH1* and *IDH2* mutations, which are typical mutations in low-grade gliomas. Wild-type *BRAF* was observed. The CpG islands of the *MGMT* promoter were found to be unmethylated, but the *TERT* promoter region showed C228T mutation, which is considered as a driver gene and is frequently mutated in GBM (Table 1).

With the diagnosis of these most common mutated genes associated with the formation of gliomas at GWH04 establishment, this cell line may be a useful tool in GBM research for the discovery of new antitumor compounds and assessment of drug sensitivity, resistance, and toxicity biomarkers, as well as identification of targeted agents showing clinical utility.

Table 1
Results of WHO recommended genetic testing for gliomas.

Tested items	Results
<i>MGMT</i> promoter methylation	Unmethylated
1p delition	Intact
19q delition	Deleted
<i>IDH1</i> R132 mutation	No mutation
<i>IDH2</i> R172 mutation	No mutation
<i>TERT</i> promoter C228T mutation	No mutation
<i>TERT</i> promoter C250T mutation	Mutated
<i>BRAF</i> V600E mutation	No mutation

Cell proliferation and soft agar colony formation assays of GWH04 cells

The proliferation rate and tumorigenicity are basic features of primary cell lines, which are closely related to their popularity in cancer research. A CCK-8 assay of GWH04 cells (P10) was performed to detect the proliferation rate, which was compared with that of U87 and GL15 cells. During the first 3 days, GWH04 grew slower than U87 and GL15, but rapidly gained speed in the following 2 days, indicating that the growth of GWH04 is density-dependent (Fig. 3a). Soft agar colony formation assays can preliminarily judge the tumorigenicity of cells in vitro. Thus, to test the clone formation capacity of GWH04 cells, we conducted parallel experiments with U87 and GL15 cell lines. The assays showed that the colony formation number of GWH04 was slightly higher than that of U87 and GL15, but was not significantly different (Fig. 3b-c; GWH04 vs. U87, $p = 0.071$; GWH04 vs. GL15, $p = 0.113$). In contrast, the average diameter of GWH04 cells was significantly smaller than that of U87 and GL15 cells (Fig. 3b, d; GWH04 vs. U87, $p = 0.004$; GWH04 vs. GL15, $p < 0.001$), indicating that this newly established cell line possesses a higher clone formation capacity, but a lower proliferation rate of anchorage-independent growth than U87 and GL15.

IC50 of TMZ, CCNU, SN-38, VP-16, 5-Fu, and oxaliplatin

TMZ is the first line chemotherapy strategy for glioblastoma treatment in recent decades¹¹, and CCNU has been demonstrated to benefit glioblastoma patients in both PFS and OS, despite increased adverse events^{12,13}. Many patients with advanced glioblastoma may have treatment with bevacizumab, irinotecan, etoposide (VP-16), 5-Fu, and cisplatin as the second line or the rescue treatment strategy, even though not all pre-clinical studies present satisfactory therapeutic results^{14–17}. In this study, GWH04 cells were exposed to TMZ, CCNU, SN-38(an active metabolite of irinotecan), VP-16, 5-Fu and oxaliplatin. The

growth rate inhibition metrics of each condition six days after drug exposure and fitted dose-response curves of different drugs are presented in Fig. 3e, and the IC50s were 856.7uM (TMZ), 136.6uM (CCNU), 13.36nM (SN-38), 3.0uM (VP-16), 17.9uM (5-Fu), 21.2uM (Ox). The promoter region of *MGMT* is unmethylated, indicating resistance to TMZ treatment. Furthermore, the IC50 of TMZ is 856.7 uM in vitro, which indicates a definite TMZ-resistance when compared with U87 (200uM), T98G (800 μM) ¹⁸. Thus, GWH04 is a primary TMZ resistant cell line.

Table 2
List of some mutated genes from WES sequencing of GWH04 cells.

Gene	Location	Mutation Type	Transcript
TP53	17p13.1	missense_variant	ENST00000269305:p.Tyr205Cys/c.614A > G
PTEN	10q23.31	stop_gain	ENST00000371953:p.Tyr315*/c.945T > A
PDGFRA	4q12	missense_variant	ENST00000257290:p.Ser478Pro/c.1432T > C
ERBB2	17q12	missense_variant	ENST00000269571:p.Ser423Gly/c.1267A > G
BRCA1	17q21.31	missense_variant	ENST00000471181:p.Glu1038Gly/c.3113A > G
NF1	17q11.2	stop_lost	ENST00000471572:p.Ter640Argext*?/ c.1918T > C
MLH1	3p22.2	missense_variant	ENST00000231790:p.Val384Asp/c.1151T > A

* refers to the last changed amino acid;

ext*? refers to the extension to an unknown amino acid.

Subcutaneous and intracranial tumorigenicity

Not every cell line can form a xenografted tumor in nude mice; for example, U251 and U87 are tumorigenic, but HS683 and A172 are not. Indeed, in a study of 34 randomly selected human glioma cell lines, only 19 (56%) were able to generate tumors in immunocompromised mice ⁸. Combined with the observation that only about 10% of GBMs will generate permanent/immortal cell lines in vitro when cultured in standard medium ⁸, it is clear that the tumorigenicity in immunocompromised mice and immortalization in vitro are distinct phenotypes.

To confirm whether GWH04 cells possess tumorigenic capacity in vivo, 3 × 10⁶ cells were injected subcutaneously into the right axilla of each mouse. Tumor-burdened nude mice and the harvested tumors observed after 45 days are shown in Fig. 4a. Then, 5 × 10⁵ cells were injected into the right frontal lobe of nude mice to confirm the intracranial tumorigenicity. After 60 days, when bioluminescence imaging indicated a definite lesion, the mouse was euthanized and the brain tissue was surgically harvested, and analyzed by H&E and IHC staining (Fig. 4b). Both mouse intracranial xenografts and primary tumor tissues of the patient expressed GFAP, Nestin, EMA, EGFR, and Ki67 (Fig. 4c). Ki67 expression indicated that the proliferation status was much higher in the mouse intracranial xenograft than in the primary

tumor of the patient (Fi. 4c), probably due to the fact that in vitro culture enriched the cells with stronger proliferative ability. Additionally, the culture system faithfully recapitulated and maintained the transcriptomic status of the primary tumor. These results indicate the substantial tumorigenicity of GWH04, thereby representing a useful tool for in vitro and in vivo GBM research.

Mutated genes associated with tumorigenicity in GWH04 cells

Apart from the several genetic factors recommended for analysis to support diagnosis and treatment, several other gene mutations are firmly related to the occurrence and development of gliomas. Ishii et al. studied tumor suppressor genes, namely *TP53*, *p16*, *p14ARF*, and *PTEN*, in 34 randomly selected human glioma cells and found that mutations and deletions were at the following frequencies: *TP53* (76.5%), *p14ARF* (64.7%), *p16* (64.7%), and *PTEN* (73.5%)⁸. Another study demonstrated that approximately 86% of GBM samples harbor at least one genetic event in the core RTK/PI3K pathway, such as in *EGFR*, *ERBB2*, *PDGFRA*, or *MET*¹⁹. To investigate the mutational status of these important genes and other associated genes in GWH04 cells, WES was performed. GWH04 was found to possess mutated *TP53*, *PTEN*, *PDGFRA*, *ERBB2*, *BRCA1*, *NF1*, and *MLH1*, and wild-type *CDKN2A*, *PIK3R1*, *PIK3CA*, *Rb1*, and *MET*. The sequencing and gene mutation data are shown in Table 2.

Chromosome karyotype of GWH04 cells

In addition to heterogeneity in the biological and molecular features of GBM, chromosomal instability (CIN) is a frequently occurring event in cancer that involves numerical abnormalities of chromosomes and large-scale structural alterations. Previous studies have demonstrated that GBM cell lines have hyperdiploid karyotypes and exhibit glioma-specific chromosomal abnormalities, such as gain of chromosome 7 and loss of chromosome 10^{4,20}. The range of possible chromosome numbers of some GBM cell lines in previous studies was sorted and presented in Table 3.

To determine whether GWH04 cells had CIN, the 10th passage cells were harvested for G-banding karyotype analysis and G-banding metaphase images were obtained. The chromosomal number ranged from 61 to 125 while counting 40 karyotypes, and the first and third quartiles were 82 and 86, respectively (Fig. 5a). Considering that reported glioma cell lines are hyperdiploid^{5,20-22} without normal diploid and hypodiploid, we conclude that hyperdiploid is a common phenomenon in glioma and glioma cell lines. Chromosome karyotype pairing showed that extra copies of different chromosomes were rather common, and aberrantly structured chromosomes presented with two unknown chromosomes in one karyotype analysis (Fig. 5b). In addition to chromosome number abnormalities, chromosomal structural abnormalities, such as broken chromosomes, double centromere chromosomes, homogeneous staining regions, and double microbodies in other karyotype analyses of GWH04 were found (Fig. 5c). These changes in overall chromosomal configuration expand our understanding of how mutations can affect the genome, as alterations in overall chromosomal structure and number also constitute types of genetic change, which must be considered as consequences of mutations. Importantly, the abnormal

chromosomes seen in these cultured cells provided the first clue that these cells may be genetically aberrant (mutants).

Table 3
Abnormal chromosome number of glioma cell lines in previous studies.

Cell line	Number of chromosomes	Number of karyotyped cells	reference
GL15	75–90	20	20
GL22	56–73	10	20
K308	54–108	100	21
ANGM-CSS	88–91	-	22
SHG39	68	1	23
LN18	70–80	-	5

Subclone analysis

Each GBM harbors considerable intratumor heterogeneity and branched evolution. Primary cell culture of the tumor tissue can maintain the heterogeneity of the derived tumor tissues without contamination by different cells or genetic drift owing to several decades of culture. To better understand the dynamic chromosome instability of GWH04, monoclonal culture from these cells and karyotype analysis of three subclones (subclone 1.4, 1.5, and 3.17) were conducted and compared. The chromosome numbers of the three subclones were not exactly the same, with a narrow range of fluctuations and more common dicentric chromosomes compared with GWH04 cells. The chromosome numbers of 40 metaphase subclone 1.4, 1.5, and 3.17 cells ranged within 59 – 83, 70 – 87, and 55 – 91, with 7.5% (3/40), 40% (16/40), and 20% (8/40) positive for dicentric chromosomes, respectively (Fig. 5d). As genetic and genomic aberrations are the primary cause of cancer, chromosome missegregation leads to aneuploidy, and aberrant chromosomes provide cancer cells with a mechanism for losing tumor suppressor loci and gaining extra copies of oncogenes, which will definitely increase the mutation rates of offspring cells.

Discussion

Cell culture and cell lines are the fundamental and most powerful tools in cancer research. They are often used in cancer biology studies and for the discovery of new antitumor compounds, drug sensitivity and resistance, toxicity biomarkers, and targeted agents showing clinical utility. However, the accumulation of genetic aberrations in cancer cell lines often occurs with increasing passage numbers, making it impossible for many preclinical studies to translate to clinical application. Therefore, establishing new

primary cancer cell lines derived from primary tumor tissues is of crucial importance for different cancer research studies.

In recent years, primary culture conditions for GBM have mainly been high-glucose DMEM or RPMI 1640 supplemented with 10% FBS for 2D adhesion culture and serum-free culture medium (neurobasal medium + B27 + EGF + FGF) for 3D suspension sphere culture, as the latter maintains more stem cell features^{23–25}. Different culture conditions were selected according to different study goals. Compared with sphere culture cell lines, adherent cultures are easy to manipulate for common experimental purposes, such as transfection, transduction, and drug screening. Adherent tumor cell lines are also amenable to growth in sphere cultures and commonly switched to sphere

cultures for specific assays. In this study, we conducted culture from 10 GBM tissues of different patients in the same culture medium (high-glucose DMEM + 10% FBS), only GWH04 from patient 4 could circumvent replicative senescence and acquire the ability to sustain unlimited proliferation in this culture medium. As the genetic backgrounds of different GBMs are not determined when establishing primary cultures from fresh patient-derived tissues, high efficiencies may result from culturing under various media conditions that differ in their combinations of growth factors.

For the identification of GWH04 cells, immunofluorescence staining of GFAP and Nestin were conducted (Fig. 2b). GFAP, a glial fibrillary acidic protein, is a marker for astrocyte activation. Although GFAP can be readily detected in most astrocytic glioma and GBM cells, only the most morphologically differentiated cells express it, whereas more primitive and anaplastic cells do not⁴. Nestin, an intermediate filament protein that is specifically expressed in neuroepithelial stem cells, is a marker of neural stem cells. It is normally expressed in the neuroepithelium during the early stage of embryonic development. For normal brain tissue and normal astrocytic cells, Nestin expression is negative. In our study, immunofluorescence staining (Fig. 2b) showed clear Nestin expression with a distinct sense of cytoskeleton fibers, which indicates firm derivation from tumor cells, whereas GFAP expression was blurry when compared with GFAP expression in cultured cells digested from pilocytic astrocytoma (stained in the same conditions) (Supplementary Fig. 2), which was positive and possessed a distinct sense of fibrous skeleton. However, this did not affect protein expression in the mouse intracranial xenografts of this cell line (Fig. 4c). Bigner et al. has showed that only two of the 15 GBM cell lines (U251 MG and U251 MG sp) yield positive immunofluorescent reactions to GFAP⁴, and another study showed that, in the early passages, about 30% GL15 and 40% GL22 cells contain GFAP immunoreactivity in the fibrils of the cytoplasm²⁰. In conclusion, a lack of GFAP expression in cultured glioma cell lines is rather common.

TP53 is a typical tumor suppressor gene associated with several biological processes, such as DNA damage repair, apoptosis, and proliferation²⁶. Indeed, among all genes in human cancer cell genomes examined to date, TP53 is the most frequently mutated gene and is mutated in the genomes of almost one-third of all human tumors²⁷. Ishii et al. have confirmed preliminary observations that the incidence of TP53 mutations is much higher in GBM cell lines (> 75%) than in gliomas (25–35%)⁸. As GWH04 also

possesses TP53 mutation, it is reasonable to consider that cells with mutated TP53 have a survival advantage in vitro in DMEM + 10% FBS cultures during cell line establishment.

TERT encodes a highly specialized reverse transcriptase, which adds hexamer repeats to the 3'-end of chromosomes²⁸. Somatic mutations of the TERT promoter region (C228T or C250T) increase telomerase activity, which leads to the preservation of telomeres, thereby allowing tumors to avoid the induction of senescence²⁹. We conducted 10 cases of primary cell cultures from different GBM tumor tissues, most of which showed slow growth and gradual aging after 5–6 passages, with the exception of GWH04 cells, which could be passaged continuously and immortalized. Accordingly, the accommodation of GWH04 cells both in vitro and in vivo may result from the combination of different mutations. With the continuous optimization of current technology and culture conditions, primary culture is expected to improve in the future.

Genome instability is a common characteristic of tumor cells. Interestingly, GWH04 was found to have chromosome number abnormalities and structural abnormalities mainly owing to chromosomal segregation errors. Karyotype analysis of several subclones illustrated the dynamic chromosome instability of these cells. The increased tolerance to chromosomal segregation errors may contribute to the association between hyperploidy and genomic complexity, and may reflect the progressive adaptation of self-renewing cells to the microenvironment in vivo and their culture conditions in vitro³⁰. Therefore, exploring relevant therapy from the perspective of aneuploidy and chromosomal abnormalities, may be effective for treating a wide range of human cancers³¹.

Tumors are usually driven by polygenetic abnormalities, with multiple aberrations in several driver genes and accompanying passenger mutations. Given the common use of several cell lines to illustrate the function and contribution of a single molecule and/or mutation to cancer, it is of great importance to learn more about the mutation background of each cell line. If a study is based on several cell lines with similar mutational landscapes, and the cell lines are of early passages with a high similarity to the primary tumor, it is expected that the transition time from preclinical to clinical research will be shortened, resulting in more precise personalized medicine.

Herein, significant genetic information and cell biological characteristics of GWH04 cells were presented. Although GWH04 cells were not able to fully recapitulate all the biological characteristics of the primary glioblastoma tissue from the patient, we believe that this cell line has extraordinary experimental utility for understanding and modeling the etiology and treatment of glioblastoma. As the poor performance of most investigational cancer drugs implies that standard preclinical disease models are faulty or improperly used³², the easy preparation and culture of pure primary cancer cells provides an innovative platform for studying cancer biology and developing personalized medicines³³. To date, this cell line has shown to be easy to manipulate for common experimental purposes, such as transfection, transduction, and drug screening, with strong stability in various experimental studies. The GWH04 cell line (first named as Fu) was deposited at BRICS and also preserved in CCTCC, and researchers can obtain this cell from either of both institutions.

Methods

Clinical history and characteristics

A 72-year-old woman was admitted to the Neurosurgery Department of Tongji Hospital in November 2016 with a 10-day history of headache and left limb weakness, and 4 days of rapid aggravation since the initial onset. Computerized tomography (CT) and magnetic resonance imaging (MRI) of the brain revealed a lesion occupying the right temporal lobe with large areas of surrounding edema (Fig. 1a-d). Gadolinium-enhanced MRI of the head showed a solid, enhancing lesion on the T1-weighted sequence, with the appearance of an irregular ring (Fig. 1e). The tumor was grossly microscopically resected during surgery, and a histological diagnosis of GBM was made according to the 2007 World Health Organization classification of tumors of the central nervous system. However, the tumor recurred rapidly, and the patient died within 3 months after surgery. Tumor tissue was acquired during the surgery for paraffin tissue embedding, liquid nitrogen cryopreservation, and primary cell culture.

Immunohistochemistry and pathological examination of glioma tissues

Immediately after resection, the tumor samples were fixed in 4% paraformaldehyde, dehydrated with an ethanol gradient, permeabilized with xylene, and embedded in paraffin. Paraffin-embedded tissue sections (4- μ m thick) were generated and stained with hematoxylin and eosin (H&E) (Fig. 1f). Continuous tissue sections were generated and immunohistochemically (IHC) stained for GFAP, NeuN, Olig2, MGMT, Nestin, EMA, Syn, c-myc, EGFR, p53, and Ki67. Antigen retrieval was performed in a microwave with citrate buffer (pH 6.0), and the inactivation of endogenous peroxidase was performed in 3% H₂O₂. Bovine serum albumin (3%) was added to evenly cover the tissue and block nonspecific antigens for 30 min at 25–28°C. Then, the slides were incubated overnight (about 15 h) at 4°C with primary antibodies at appropriate dilutions. The next day, the sections were incubated with biotin-labeled secondary antibodies for 50 min and the final signals were developed using 3,3'-diaminobenzidine substrate (R&D Systems, Minneapolis, MN, USA). The sections were analyzed by optical microscopy after counterstaining with hematoxylin. All procedures and subsequent histological diagnoses were performed in the Pathology Department of the Tongji Hospital.

Primary cell culture and authentication

The tumor specimen (< 1 cm³) was obtained shortly after surgery and rinsed twice with phosphate-buffered saline (PBS) to wash out residual red blood cells. The specimen was prepared as a single cell suspension by mechanical dissociation (1–2 mm) with sterile scissors, treated with 4 mL of 0.125% trypsin⁵, and agitated with a horizontal shaker (180 rpm) until the tissue granules became smaller. All suspensions were passed through a sterile filter with a 200- μ m pore diameter and centrifuged (300 g, 5 min). The cell pellets were resuspended in complete medium (10% fetal bovine serum [FBS] + Dulbecco's Modified Eagle Medium [DMEM]) and seeded on a 25 cm² culture flask as adherent culture in a humidified incubator with 5% CO₂ at 37°C.

During the first five passages, only half of the medium was changed each time. When a monolayer of primary tumor cells was formed and reached 90% confluence, they were passaged after 0.25% trypsin digestion, and cultured at 1:3–1:5 dilution in a new flask. After subsequent passages, as with the U251 and U87 cell lines, all stably passaged cells were viably frozen in medium plus 10% dimethyl sulfoxide and stored at -80°C ⁵.

For authentication, DNA from culture cells and primary tumor tissue was isolated using AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, Union City, CA, USA), and 21 loci STRs were examined and compared with the corresponding STR profile of the tumor tissue. After bacterial and mycoplasma contamination tests were confirmed negative, the GWH04 cell line (first named as Fu) was deposited at the Bioresearch Innovation Center, Suzhou (BRICS), China, and also preserved in the China Center for Type Culture Collection (CCTCC), Wuhan, China.

Detailed information was given to the patients or the patients' legal guardians and informed consent were written prior to surgery. All the samples were obtained from the Department of Neurosurgery, Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China) after written informed consent and according to the protocol approved by the Research Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (serial no. TJ-IBR20181111). All the procedures involving human samples was in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Immunofluorescence assay of GWH04

Single-cell suspensions of GWH04 were seeded into 24-well plates with round glass coverslips. After 24 h, the cells were fixed with 4% paraformaldehyde at room temperature ($25-28^{\circ}\text{C}$) for 15 min, permeabilized with 0.25% Triton X-100 for 5 min, and blocked with goat serum (5%) for 1 h. Primary antibodies against GFAP and Nestin were added and incubated overnight at 4°C . Cells were rinsed with PBS, and fluorescence-labeled secondary antibodies were added and incubated at room temperature for 1 h. The cells were then stained with DAPI, rinsed with PBS, sealed with a mounting medium, and analyzed using a fluorescence microscope (Olympus, Tokyo, Japan). To compare GFAP levels, cells from pilocytic astrocytoma tissue were also stained for GFAP under similar staining conditions as GWH04.

Cell proliferation and soft agar clone formation assay

Cells (GWH04, U87, and GL15) were seeded onto 96-well plates at 2×10^3 cells per well in 100- μL suspensions in triplicates and maintained in complete culture medium for 5 days. The cell proliferation rate was measured using the Cell Counting Kit-8 assay (CCK-8, KJ800; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

For soft agar assay, 0.5% Basal Medium Eagle (BME) agar, containing 10% FBS, 2 mM L-glutamine, and 25 $\mu\text{g}/\text{mL}$ gentamicin, was added to 6-well plates (3 mL/well), with triplicates per cell line. The plates were then placed in an incubator for 1 h to coagulate the semi-solid medium. Subsequently, GWH04 cells were resuspended at a concentration of $9 \times 10^3/\text{mL}$ in 0.33% BME agar containing 10% FBS, and seeded

at 1 mL/well. The cultures were maintained at 37°C in a 5% CO₂ incubator for 14 days, and the cell colonies were observed via microscopy. The number of colonies and their relative diameters were recorded and analyzed.

Half maximal inhibitory concentration (IC₅₀) of temozolomide (TMZ), lomustine (CCNU), irinotecan (SN-38), etoposide (VP-16), 5-fluorouracil (5-Fu), and oxaliplatin

GWH04 cells were seeded onto 96-well plates at 3×10^3 cells per well in 100 μ L suspensions in triplicate for every drug concentration and maintained in complete culture medium for 24 h. Then the culture medium was changed with culture mediums containing different drug concentrations and maintained for 6 days. The culture medium was changed on the third day. Subsequently, cell viability was assessed using the Cell Counting Kit-8 assay (CCK-8, KJ800; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Drug inhibition curves were plotted and IC₅₀ was calculated using GraphPad Prism 5.

Detection of IDH mutational status, 1p/19q codeletion, MGMTp methylation, TERTp mutation, and BRAFV600E mutation

DNA from cultured GWH04 cells and liquid nitrogen-frozen tumor tissues of the patient were extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Next-generation sequencing was conducted to analyze the mutational status of isocitrate dehydrogenase 1/2 (IDH1/2), 1p/19q codeletion, telomerase reverse transcriptase (TERT) promoter mutation (C228T or C250T), and BRAF mutation (V600E). As pyrosequencing is a highly accurate method to analyze the changes at one or more CpG sites in the methylated sequence, the detection of the methylation status of the O6-methylguanine-DNA methyltransferase (MGMT) promoter of GWH04 cells was conducted by Genetron (Beijing, China) using PyroMark Q24 sequencer (Qiagen), which includes a complete software package for analyzing CpG site methylation and built-in controls for confirming the completeness of bisulfite processing.

Determination of glioma cell tumorigenicity

Male BALB/c athymic nude mice (4–5 weeks of age) were purchased from Hunan Silaike Jingda Laboratory Animal Co. (Changsha, China) and housed under specific pathogen-free conditions in a temperature- and humidity-controlled environment. First, GWH04 cells were harvested and injected subcutaneously into the right axilla of each mouse (3×10^6 cells/mouse) to test the tumorigenicity. Subsequently, the intracranial tumorigenic capacity was assessed as follows. A short longitudinal incision was made in the scalp to expose the calvarium after anesthesia. Then, a burr hole was made 0.5 mm posterior to the bregma and 1.5 mm to the right of the sagittal suture. A Hamilton syringe was introduced at a depth of 2 mm below the brain surface, and 5×10^5 tumor cells (stably expressing luciferase) were slowly injected into the brain. Sixty days after inoculation, bioluminescence imaging (BLI) was conducted, the mouse was euthanized, and the brain tissue was surgically harvested, measured, fixed in 4% paraformaldehyde overnight, and embedded in paraffin. H&E and IHC staining (for

GFAP, Nestin, EMA, EGFR, and Ki67) was conducted and the results were compared with those for the respective stained patient tumor tissue.

All procedures in the animal experiments were approved by the by the Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (serial no. TJ-A20161206) and were performed in accordance with ARRIVE guidelines³⁴ (<https://arriveguidelines.org>). All surgeries were performed in accordance with the relevant guidelines and regulation and under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

Whole-exome sequencing (WES) of GWH04 cells

DNA from GWH04 cells (15th passage) was extracted as described above. Library construction and whole-exome capture of genomic DNA were performed using the SureSelectXT Human All Exon V6 (Agilent) and MGIEasy Exome Universal Library Prep Set-V1.0. The captured DNA was sequenced on an MGISEQ 2000 platform (BGI-shenzhen, china) with 150-bp paired-end sequencing. The human genome data of Hg19 from the UCSC Genome Browser (<http://genome.ucsc.edu/>) was used as a reference and common mutated genes in GBM patients were analyzed in the cell line.

Chromosome karyotype analysis

Harvesting for metaphase chromosome preparations was performed by treating exponential growth phase cells with 0.25 µg/mL colchicine for 3 h. Then, enzymatic dispersal was performed with trypsin/EDTA and the preparation was centrifuged at 1,000 g for 6 min. Preheated 0.075 mM KCl hypotonic solution was added for 15 min at 37°C and fixed with 3:1 methanol-acetic acid (v: v). The pellets were fixed twice at room temperature for 10 min, and 500 µL of stationary liquid was added to resuspend the cells. Afterward, cell droplets were spotted onto clean microscope glass slides that had been soaked in iced water. The slides were immediately heated over an alcohol lamp for 2 s to allow the chromosomes to spread out. Chromosome specimens were stained with Giemsa, and the chromosome numbers of M phase cells were counted using an oil immersion lens (100×) under a microscope (Nikon, Tokyo, Japan). Karyotypes were determined by arranging all the photographed metaphases. The chromosomes were classified according to the International System for Human Cytogenetic Nomenclature²⁰.

Declarations

Author contributions statement

D.G. and B.W. discussed the project and designed the research. F.C. and F.M. put forward the ideas of this article, conducted the primary culture and wrote this article. D.G. revised the manuscript. F.C. and X.W. performed the karyotype analysis and animal experiments. Y.L. and P.P. conducted the acquisition and data analysis from CCK-8, soft agar colon formation assay. C.H. collected the clinical samples and

conducted the follow-up records. S.X. and B.W. performed the pathological examination of tumor tissue and xenograft. All authors read and approved the final manuscript.

Data availability statement

The raw data supporting the conclusions of this manuscript will be made available by the authors without undue reservation. The WES-seq dataset generated during the current study is available in the NCBI repository and the accessions number is PRJNA806683.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

Figure 1

Preoperative imaging and pathologic features of the tumor. (a) Axial CT scan revealed a space occupying lesion in the right temporal lobe. (b-e) Axial MRI scan of T1-weighted, T2-weighted, T2-FLAIR and gadolinium-enhanced T1-weighted images, all demonstrated the occupied lesion in the right temporal lobe. (f) H&E stain of the resected tumors, showed numerous nuclear atypia (blue arrow) and mitoses (red arrow).

Figure 2

Bright field morphology, immunofluorescent staining and STR identification of GWH04. (a) Bright field morphology of GWH04 cells in the 1st, 2nd, 10th and 50th passage (P1, P2, P 10, P50; Scale bars, 100 μm). (b) The immunofluorescence staining of GFAP and Nestin. DAPI is a nuclear dye used as counterstain in immunofluorescence (400×). (c) STR loci in tumor tissue and GWH04 cells and corresponding identification map.

Figure 3

The proliferation rate, anchorage-independent growth capacity and chemotherapy sensitivity of several drugs of GWH04. (a) Analysis of proliferation rates with CCK-8 in GWH04, U87 and GL15 cells. (b) Anchorage-independent proliferation with colony formation assays of GWH04, U87 and GL15 cells.

Representative images of soft agar colonies in each cell line are presented. Scale bars, upper panel, 200 μm ; lower panel, 50 μm . (c) Quantitative analysis of colony numbers per well of each group ($n = 3$ independent wells per cell line, Data represent the mean $\pm$ SD of three independent experiments). There was no statistical difference in the number of clones formed among the three cells. n.s., no significance. (d) Quantitative analysis of 10 different clones' diameters showed GWH04 cells formed significantly smaller clones than that of U87 and GL15 cells ($n = 10$ biologically independent colonies per cell line). $**p < 0.01$, $***p < 0.001$. (e) The corresponding dose-response curves of GWH04 cells exposed to increasing concentrations of TMZ, CCNU, SN-38, VP-16, 5-Fu and oxaliplatin, and the different IC50s of each drug were presented.

Figure 4

The tumorigenic capacity of GWH04 cells. (a) Images of nude mice bearing GWH04 subcutaneous xenografts, and the harvested tumors were presented in the lower panel. (b) Bioluminescence imaging and H&E staining of the mouse brain tissue obtained after 60 days post-implantation. (c) Tumor tissues and the primary glioblastoma tissue of the patient were sectioned and immunostained for detecting the expression of GFAP, Nestin, EMA, EGFR and Ki67. Representative images are shown (Scale bar: 100 μ m).

Figure 5

Karyotype analysis of GWH04 cell line and chromosomal instability presented in three subclones of GWH04 cells. (a) The chromosomal numbers of 40 different G-banding metaphase cells. (b) Chromosome karyotype pairing in one karyotype analysis showed extra copies of different chromosome, one broken chromosome (blue arrow) and two unknown chromosomes (red arrow). (c) Several other karyotype analyses of GWH04 cells demonstrated chromosomal structural abnormalities. Arrows indicated abnormal chromosomes (broken chromosome, double centromere chromosome, homogeneous staining region, and double microbodies). (d) Chromosome number and frequently occurred double centromeric chromosome in three different monoclonal subclones (subclone 1.4, subclone 1.5 and subclone 3.17) of GWH04 cell line.

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