Cell line-based xenograft mouse model of paediatric glioma stem cells mirrors the clinical course of the patient

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Abstract

The leading cause of cancer-related mortality among children is brain tumour, and glioblastoma multiforme (GBM) has the worst prognosis. New treatments are urgently needed, but with few cases and clinical trials in children, pre-clinical models such as patient-derived tumour xenografts (PDTX) are important. To generate these, tumour tissue is transplanted into mice, but this yields highly variable results and requires serial passaging in mice, which is time-consuming and expensive. We therefore aimed to establish a cell line-based orthotopic mouse model representative of the patient tumour. Glioma stem cell (GSC) lines derived from paediatric GBM were orthotopically transplanted into immunodeficient mice. Overall survival data were collected and histological analysis of the resulting neoplasias was performed. Genome-wide DNA methylation arrays were used for methylation and copy-number alterations (CNA) profiling. All GSC lines initiated tumours on transplantation and the survival of the mice correlated well with the survival of the patients. Xenograft tumours presented histological hallmarks of GBM, and were also classified as GBM by methylation profiling. Each xenograft tumour clustered together with its respective injected GSC line and patient tumour based on the methylation data. We have established a robust and reproducible cell line-based xenograft paediatric GBM model. The xenograft tumours accurately reflected the patient tumours and mirrored the clinical course of the patient. This model can therefore be used to assess patient response in pre-clinical studies.

Introduction

Brain tumours are the most common solid tumour in children and paediatric high-grade gliomas, including glioblastoma multiforme (GBM), are among the most devastating forms with a 5-year survival of less than 20% (1). Recent studies using whole genome sequencing and DNA methylation analysis have shown that paediatric GBM differs from adult GBM in such ways that they cannot be viewed as the same disease (2, 3). Point mutations in genes such as IDH and TP53, and methylated O6-methylguanine DNA methyltransferase (MGMT) are commonly detected in adult tumours but not in paediatric tumours. Instead, other mutations affecting the methylation pattern and chromatin structure have been observed, such as the hallmark missense mutations in the genes H3F3A and HIST1H3B (4). Paediatric tumours also harbour less copy-number alterations (CNA) than adult GBM, although some alterations occur. These include amplification of epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor alpha (PDGFRα), deletions of retinoblastoma 1 (RB1), phosphatase and tensin homolog (PTEN) and cyclin-dependent
kinase inhibitor 2A/B (CDKN2A/B) (5–7). Even though collection of paediatric tumour tissue, through large collaborations like the Children’s Brain Tumour Tissue Consortium (8), allows for important studies of tumour cells, there are few in vitro and in vivo models. The majority of published in vitro models are cultured with serum (9), which does not retain the features of the tumours (10, 11), or in serum-free media as neurospheres (12, 13). Previously reported in vitro models on paediatric high-grade glioma include orthotopically injected tumour tissue, so called patient-derived tumour xenografts (PDTX) (14), and subcutaneously injected cell lines (12). However, reports of accumulating genetic alterations when culturing human tumour cells in mice due to a selection pressure from the host suggest that improvement of the PDTX model is needed (15). The transplantation of cell lines is more reliable and reproducible since the number of tumour-initiating cells is known; however, subcutaneous transplants do not resemble the environment of the brain. So far, there are no well-described cell line-based orthotopic xenograft models for paediatric GBM. Such a model would be more effective than PDTX models as cells are not passed in mice, thus reducing the number of animals needed as well as cost and time. We have recently published a study, describing the establishment and characterization of patient-derived glioma stem cell (GSC) cultures maintained under serum-free conditions (16). Here, we extended this study with thorough characterization of orthotopic cell line-based xenografts in immunocompromised mice initiated from these GSCs. The tumours that formed resembled the original tumours, and the disease course of the mice reflected the clinical course of the patients. This model is repeatable, reliable, stable and suitable for functional studies in the search of novel tumour markers and new treatments.

Material and methods

This study was carried out in accordance with guidelines and regulations approved by the regional ethics review board of Gothenburg (Dnr 604-12) and the Swedish Board for Agriculture (Dnr 10-2015). Signed informed consent was obtained from the children’s parents before tumour collection. Six paediatric GBM samples were collected at the Sahlgrenska University Hospital (Gothenburg, Sweden); all were primary tumours except for one relapsed tumour (GU-pBT-10). For patient data, see Table 1.

It should be noted that the mice in the study were untreated, whereas the patients received treatment. Treatment included neurosurgery, radiotherapy and chemotherapy in all cases, most commonly temozolomide in combination with bevazicuband, but other chemotherapy combinations were also used. The sole long-term survivor (GU-pBT-15), initially diagnosed as a primitive neuroectodermal tumour (CNS-PNET), received both focal and craniospinal irradiation followed by chemotherapy (mainly ifosfamide, carboplatin and etoposide) and valproic acid.

GSC cultures

Primary GSC cultures were established from the paediatric GBM samples collected at Sahlgrenska University Hospital during 2013–15 and have been previously described (16). Cell cultures were named accordingly: GU-pBT-7, GU-pBT-10, GU-pBT-15, GU-pBT-19, GU-pBT-23 and GU-pBT-28, and they refer to BPC-A7, BPC-B0, BPC-B5, BPC-B9, BPC-C3 and BPC-C8 in Wenger et al. (16). Cells were transplanted in passage 9–11 except for GU-pBT-23 transplanted in passage 19. The cell lines were tested and authenticated by short tandem repeat profiling (IdentitCel1, Aarhus University Hospital) in 2016 and had no match to cell lines in DSMZ or ATCC cell banks but to each other. The cell lines were also tested at regular intervals by single-nucleotide polymorphism profiling and compared with the primary tumour and earlier passages. All cell lines were tested negative for mycoplasma in higher passages by GATC Biotech.

Immunohistochemistry of xenotransplanted mouse brain

Brains were embedded in paraffin and sectioned (5 μm) using a micrometre. Sections were deparaffinized, heat-induced antigen unmasked (with citrate buffer pH 6.0) and stained according to Vecta Stain ABC kit (Vector) using rabbit monoclonal Nestin (human specific), ab105389 Abcam, 1,300; rabbit monoclonal Ki-67, ab92742 Abcam, 1:500; rabbit polyclonal glial fibrillary acidic protein (GFAP) Z 0334, Dako, 1:50. For histology, haematoxylin and eosin (H&E) staining was used.

Table 1. Patient data.

| Patient-ID | Gender | Age at diagnosis (years) | Location of the tumour | Primary or relapse | Outcome (days after diagnosis) |
|------------|--------|--------------------------|------------------------|-------------------|-------------------------------|
| GU-pBT-7   | Male   | 4.2                      | Right hemisphere       | Primary           | DOD (325)                     |
| GU-pBT-10  | Male   | 10.4                     | Right hemisphere       | Relapse           | DOD (312)                     |
| GU-pBT-15  | Female | 12.5                     | Brain stem             | Primary           | AWD (1238)                    |
| GU-pBT-19  | Male   | 6.2                      | Right hemisphere       | Primary           | DOD (485)                     |
| GU-pBT-23  | Female | 2.9                      | Left hemisphere        | Primary           | DOD (190)                     |
| GU-pBT-28  | Female | 11.1                     | Pons (cerebellopontine angle) | Primary          | DOD (237)                     |

AWD, alive with disease; DOD, dead of disease.
Statistical methods
Methylation array data were analysed using R packages ChAMP and minfi. Probes for CpG sites on the X and Y chromosomes or close to a known single nucleotide polymorphism (20) were removed as well as probes present only on either HumanMethylation450BeadChips or Infinium MethylationEPIC BeadChips. In addition, probes that were detected using the Infinium MethylationEPIC BeadChip on DNA from the mouse neural progenitor cell line were removed, resulting in 250 438 probes that were kept for further analysis.

Hierarchical clustering was performed on probes with a standard deviation across samples above 0.25, using Euclidean distance metric and complete linkage. Differentially methylated probe analysis was performed on paired patient-xenograft differences, using differentially methylated probes with adjusted P-value <0.01 (Benjamini-Hochberg) and mean difference in β > 0.51. CNA were inferred from the methylation array data, using the R package conumer.

Results
The survival time of transplanted mice correlates significantly with the survival of the patients
GSC lines derived from six patients (16) were orthotopically transplanted into immunocompromised mice. Survival time of patients and xenotransplanted mice was compared, and patient tumours, GSC lines and xenograft tumours were analysed for DNA methylation, CNA and classified with MethPed. Tumour take rate was close to 100% (a tumour was formed in 29/30 transplanted mice). The survival time for the mice in each group was close in time, as shown in the Kaplan-Meier curve (Figure 1A). The mean survival time of the mice in each group was significantly correlated to the survival of the patients, from whom the GSC cultures were established (Pearson correlation coefficient, r = 0.79, P-value = 3.5 x 10^-7; Figure 1B).

The morphology and methylation-based diagnosis of xenograft tumours resemble patient GBM
HE staining of patient tumours was performed at Sahlgrenska University Hospital and has partly been described (16). The tumour tissue exhibited characteristic GBM morphology with diffuse infiltration of anaplastic tumour cells showing high frequency of atypical mitoses and endothelial proliferation. The tumour cells frequently formed secondary structures of Scherer as perivascular proliferation and perineuronal and leptomeningeal accumulation. The tumours had variable expression of GFAP, strengthening the glial origin of the tumour, and a high number of proliferating cells (assessed by Ki-67). The samples were classified as GBM using the methylation-based classifier MethPed (16, 21). We next performed HE staining on the xenograft tumours. All GSC lines derived from patient GBM tumours gave rise to infiltrative tumours of human origin in immunocompromised mice (Figure 2). The xenograft tumour cells exhibited comparable growth patterns as the patient’s tumour cells with related morphology as described earlier (Figure 3A). The GSC culture GU-pBT-10 formed a focal tumour at the place of injection and showed limited infiltration of the surrounding brain parenchyma and less diffuse axonal growth. In contrast, the remaining GSC lines demonstrated a gliomatosis cerebri-like diffuse growth pattern. In the GU-pBT-19 xenograft, noticeable peritumoral oedema was present. Proliferation analysis using Ki-67 showed very high proliferation in all xenografts (Supplementary Figure 1). To additionally strengthen and confirm the diagnosis, we used our MethPed classifier (21), which diagnosed all xenografts as GBM with similar classification scores as the respective patient tumour (Figure 3B).

The methylome of the xenograft tumours is stable
Next, we performed hierarchical clustering on all β-values, measured with Illumina methylation BeadChips, with a standard deviation across samples above 0.25 (27 500 sites). Each group of samples, including patient tumour, GSC line and xenograft tumour, clustered together (Figure 4A). This indicates that the cells retain their characteristic methylation pattern in vitro and in vivo. Less than 3% of the studied CpG sites changed methylation status, defined as an absolute difference in β-value between samples above 0.51 (22), between either xenograft and patient tumour or cell line, strengthening the stability of the model (Figure 4B). We also looked for differentially methylated probes between paired xenograft and patient tumours. Only 127 CpG sites differed between the groups with a mean difference in β > 0.51, adjusted P-value <0.01.

Key glioma genomic alterations are stable in the xenografts
We used the methylation array data to generate copy-number profiles for the samples (Supplementary Figure 2). In general, there was a good agreement between the patient tumour and the formed xenograft tumour, although some alterations were noted. EGFR and KRAS amplifications present in most patient
tumours were also present in the corresponding xenograft tumours, as were deletions of the tumour suppressor genes RB1, PTEN and CDKN2A/B. Amplification on chromosome 4, where PDGFRA is located, was present in one of the patient tumours and in the corresponding xenograft tumour. Loss of chromosome 3 and 7 found in patient tumours was also identified in the
corresponding xenografts. Some alterations were found de novo in the xenograft tumours. These were also detected in the GSC cultures that were transplanted, suggesting that these alterations were present already in the selected stem cell population.

**Discussion**

Recent efforts in profiling the genome and epigenome of paediatric brain tumours have led to DNA methylation-based classification and subgrouping of GBM tumours (3, 23). With new potential therapeutic targets, therapy can be designed more rationally, considering the diverse molecular features of these tumours (24). Genetically stable in vivo models are needed to pre-clinically test new drugs. In our cell line-based orthotopic xenograft model, we used well-characterized paediatric GSC lines, previously shown to be stable in culture; retaining the DNA methylome and CNA commonly found in the primary tumours (16). Here, we demonstrate that these GSCs are also stable in mice, initiating invasive tumours with a GBM-like growth pattern; anaplastic cells, atypical mitoses and secondary structures of Scherer. Tumour take-rate of engraftments is close to 100% and the cohort of mice injected with each cell line die within a very tight time frame, demonstrating accurate repeatability. This is in contrast to PDTX models, where a small portion of a heterogeneous tumour containing unknown numbers of cancer stem cells (25) results in large variabilities between animals and an uneven engraftment rate (26), or even failure of tumour growth (27). Xenograft tumours from PDTX models have also been shown to be influenced by the mouse microenvironment, rapidly acquiring CNA over multiple passages in animals as well as losing CNA present in the patient tumour (15). In comparison, although long-time cell cultures can accumulate genetic alterations, key glioma CNA found in patients were retained in our cell line-based mouse model, and the xenograft tumours presented a stable methylome with similar methylation pattern as the patient tumour. Less effort in terms of animal care and surgical procedures are needed and the number of animals is reduced, which is preferable both from an ethical and economical point of view.

Importantly, this thorough characterization of the xenograft tumours and strong correlation to the patient survival add a dimension of clinical usefulness to previous published work on our cell culture system. This is, to the best of our knowledge, the first paediatric GBM model that mimics the clinical course of the patient. It further enables studies to expand our understanding of the pathology and molecular driving forces behind paediatric GBM; an important step towards finding new treatments and improving the prognosis for affected children.

**Supplementary material**

Supplementary data are available at Carcinogenesis online.

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