A Comprehensive Approach to Patient-individual Glioblastoma Multiforme Model Establishment

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Abstract

Patient-individual tumor models for Glioblastoma Multiforme (GBM) are important not only for basic and translational research but also for the development and improvement of optimal and individualized treatment strategies. The model that has gained widest acceptance is the primary cell culture model. The laborious and time consuming process is rewarded with a relative high initial success rate (about 60%). We here describe and evaluate an extended biobanking methodology to simplify sample collection and model establishment. GBM resection specimens were collected ad hoc, partially prepared fresh for modeling, snap frozen for molecular testing and frozen down vitally.

The established models were subject to subsequent detailed characterization in direct comparison to the patients’ tumors. Generally, molecular characteristics such as mutations, gene amplifications and epigenetic alterations were maintained in the models. Immortality, neuronal origin and stem cell characteristics of the cell lines could be demonstrated. Extensive drug sensitivity screens were performed. These well-defined patient-individual models are ideal for establishment of individualized therapy approaches and enable testing of immunological strategies.

Our extended biobanking procedure facilitates collection, long-time storage and propagation (modeling) of clinical GBM specimens (potentially also from distant centers) for basic research, (pre-) clinical studying of novel therapies and individual response prediction.

Keywords: Glioblastoma multiforme; In vitro models; Molecular characterization; Drug testing; Phenotyping

Abbreviations: BTSC: Brain Tumor Stem Cell; CGH: Comparative Genomic Hybridization; EGFR: Epidermal Growth Factor Receptor; GBM: Glioblastoma Multiforme; IL-13Rα: Interleukin 13 receptor alpha; MGMT: O6-methylguanine-DNA methyltransferase; TAA: Tumor Associated Antigen; TMZ: Temozolomide; WHO: World Health Organization

Introduction

Glioblastoma Multiforme (GBM) is the most frequent neoplasm of the brain, characterized by a very dismal prognosis [1,2]. Despite multimodal treatment consisting of resection followed by irradiation and chemotherapy with temozolomide (TMZ), median survival only ranges from 9 to 12 months [3]. A highly invasive phenotype usually leads to local recurrence even after macroscopically complete resection [4]. Different approaches have been undertaken to classify and better stratify (e.g. with regard to optimal treatment strategies) these tumors. The World Health Organization (WHO) classification differentiates tumors according to their histology, morphology and degree of malignancy (WHO grade I-IV); GBM are astrocytic tumors WHO grade IV [5]. Those are divided into primary GBM appearing de novo as grade IV tumors and secondary GBM that progress from low grade to grade IV tumors [6]. Primary GBM are characterized by amplification and/or mutation of the epidermal growth factor receptor (EGFR), whereas secondary tumors typically show mutations in the genes IDH 1 and 2 [6]. The most recent approach classifies GBM by the origin of the tumor initiating cell type: pro-neural (oligodendrocytic cells), neural (neurons), mesenchymal (astroglia, microglia) and classical (astrocytic cells) GBM [7]. Especially the latter classification takes a variety of molecular characteristics into account, which is enabled by increasingly extensive molecular pathological profiling. Key analyses include methylation status of the O6-methylguanine-DNA methyltransferase (MGMT) promoter and mutational profiling of IDH 1 and 2, TP53, PTEN, RB-1, NF-1 and EGFR [8,9]. The mutation of EGFR coincides with an amplification of the receptor [10,11]. Finally, chromosomal changes such as loss of or loss of heterozygosity at 10q as well as loss of 1p and 19q (alone or as co-deletion) are common GBM features [8,12].

Recently, Duarte and coworkers affirmed this concept by describing a gene signature (IFN/STAT1) in the proneural subtype which may be responsible for poor prognosis due to chemotherapy and/or radiation resistance in these tumors. Their results might have strong implications both for better prediction of survival outcome and for improved understanding of GBM subtype-specific tumor progression mechanisms and treatment response [13].
Consequently, the heterogeneity of GBM must be considered in drug development and preclinical testing. Patient individual tumor models provide ideal material for such studies. These individual models are likely to allow the most accurate response and resistance prediction outside the patient. The high prediction precision of individual carcinoma models was demonstrated by Voskoglou-Nomikos and colleagues as well as by Fiebig and co-workers with 90% and even 97% accuracy for prediction of response and resistance, respectively [14,15].

We here aimed at the establishment of a comprehensive collection of GBM cell models out of a consecutive series of clinical cases. In vitro cell line establishment was performed in parallel with in vivo engraftment into immunodeficient mice. And this model collection was subsequently deeply characterized taking the above described molecular classifications and markers well into account.

Materials and Methods

Tumor specimen collection and cryopreservation

Between August 2009 and October 2012, 42 clinical samples from patients with GBM WHO grade IV (Table 1) were collected from the Neurosurgery department at the University Medicine Rostock. Prior informed consent was obtained in written form from all patients, and all procedures were approved by the institutions’ Ethics Committee (reference number: A 2009/34) in accordance with general accepted guidelines for the use of human material. Resection specimens of GBM tumors (n=42) were received sterile and freshly from surgery. Tumor tissue samples were snap frozen in liquid nitrogen and stored in the gas phase above liquid nitrogen. Additionally, tumor tissue cubes (3 × 3 × 3 mm) were frozen vitally. For this procedure, tumor pieces were cut with a sterile scalpel blade, and 4 tumor pieces were transferred into one sterile cryo-tube in 1.5 ml freezing medium (fetal calf serum containing 10% DMSO), sealed in a freezing container (Nalgene, Rochester, USA), and placed immediately at -80°C. Until thawing, tubes were kept at -80°C (for a maximum of 6 weeks) or, after overnight cooling, transferred into a nitrogen tank (for longer storage periods). For subsequent modeling procedures, cryopreserved tumor pieces were thawed at 37°C.

Xenografting into immunodeficient mice

Tumor xenografting was done by one of the following approaches: (I) xenografting of fresh tumor pieces on the day of surgery (n=10); (II) xenografting of tumor pieces after cryopreservation (n=36); and (III) re-transplantation of xenografts (n=3). Tumor pieces were implanted subcutaneously bilaterally into the flanks of six to eight week old female mice under short term ether anesthesia. We used NMRI nu/nu mice for the xenograftings. Mice were kept in the animal facilities of the University Medicine Rostock and maintained in specified pathogen-free conditions. Animals were exposed to 12 h light/12 h darkness cycles and standard food and water including antibiotics (Co-trimoxazol) ad libitum. Their care and housing were in accordance with guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Care and Use, Bethesda, Maryland). Photographs were edited with Photoshop CS3 (Adobe, München, Germany).

Phenotypic characterization (microphotography)

Cells were cultured in T25 flasks to a confluence of 60-80% and photographed using the AxioVision 4.8.2 software (Carl Zeiss, Jena, Germany). Photographs were edited with Photoshop CS3 (Adobe, München, Germany).

Growth kinetics

Cells (5 × 10⁶ cells) were plated in 5 ml media in quintuplicate T25 culture flasks per cell line and allowed to attach and grow for 48 h. Cells were detached by trypsinization and the amount of vital cells was assessed by trypsin blue staining using a Neubauer chamber. One flask was counted every 24 h for five consecutive days.

Isolation of nucleic acids

Genomic DNA (gDNA) from snap frozen tumor tissue and cell culture cell pellets (3 × 10⁶ cells) was isolated using the Wizard Genomic DNA Purification Kit (Promega, Mannheim, Germany) according to the manufacturer’s instructions. Total RNA from cell culture pellets (3 × 10⁶ cells) was isolated using the EURs Gene MATRIX Universal RNA Purification Kit (EURx, Gdańsk, Poland) according to the manufacturer’s instructions. Concentration of isolated nucleic acids was determined with the NanoDrop1000 (Thermo-Scientific, Wilmington, USA).

cDNA synthesis

2 µg total RNA was used for reverse transcription applying the High Capacity cDNA Reverse Transcription Kit according to the manufacturer’s instructions (Applied Biosystems, Carlsbad, CA, USA).

Molecular Characterization

MGMT promoter methylation

For analyzing the MGMT promoter methylation, the MethyLight method was applied. Briefly, gDNA was subject to bisulfite conversion using the Epitect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. A primer/probe combination specific for methylated MGMT promoter sequence was used, with the SensiFast Probe Kit (Bioline, Luckenwalde, Germany). CpG Methylation (Sssl) (New England Biolabs, Frankfurt, Germany) treated DNA served as calibrator, since it is considered to be fully methylated. The collegenase gene 2A1 (COL2A1), was used as endogenous control. The percentage of methylated reference (PMR) value was calculated by dividing the MGMT/COL2A1 ratio of the sample by the MGMT/ COL2A1 ratio of the Sssl-treated DNA, and multiplying by 100. Samples with a PMR value >4 were considered as methylated [16]. All reactions were performed in triplicates. Used Primers are listed in Table 2.
The table summarizes patient information on sex (F=female; M=male), age at time point of resection in years, histological diagnosis including WHO grading in parentheses, tumor localization (L=left hemisphere; R=right hemisphere) and survival (†=patient died; bold=patients still alive on January 25th 2013; ?=no information available for > 6 months) in months after resection. Successful cell line establishment is indicated by a C, cell lines characterized in the following are bold and establishment of xenografts by an X. The X* indicates that a xenograft could be established but was lost subsequently.

| Sample ID | Sex | Age | Diagnosis                  | Localization             | Survival | Models |
|-----------|-----|-----|----------------------------|--------------------------|----------|--------|
| HROG02    | M   | 68  | GBM (IV)                   | R; parietoccipital       | † 7      | C      |
| HROG03    | M   | 50  | Anaplastic Oligodendroglioma (III) | R; parietal              | ? 9      |        |
| HROG04    | F   | 53  | Relapsed GBM (IV)          | R; frontal               | † 13     | C      |
| HROG05    | F   | 60  | Relapsed GBM (IV)          | L; temporal              | † 3      | C, X   |
| HROG06    | M   | 53  | GBM (IV)                   | L; frontal               | † 8      | C, X   |
| HROG07    | M   | 55  | Relapsed GBM (IV)          | R; temporoparietal       | † 6      | C      |
| HROG08    | M   | 47  | Relapsed GBM (IV)          | R; frontal               | ? 29     | C      |
| HROG09    | M   | 66  | Anaplastic Astrocytoma (II-III) | L; temporal              | 33       |        |
| HROG10    | M   | 74  | GBM (IV)                   | R; temporal              | † 7      | C      |
| HROG11    | F   | 54  | GBM (IV)                   | L; frontal               | 30       | C      |
| HROG12    | M   | 64  | GBM (IV)                   | R; frontoparietal        | † 5      | C, X   |
| HROG13    | F   | 77  | GBM (IV)                   | L; temporal              | † 8      | C, X*  |
| HROG14    | F   | 81  | Subependymoma (I)          | IV; ventricle            | † 3      |        |
| HROG15    | M   | 56  | GBM (IV)                   | R; parietal              | 23       | C      |
| HROG16    | M   | 53  | GBM (IV)                   | R; parietal              | † 26     |        |
| HROG17    | M   | 70  | Relapsed GBM (IV)          | L; parietooccipital      | † 3      | C, X   |
| HROG18    | M   | 71  | Relapsed Oligoastrocytoma (II) | cerebrum                | ? 7      |        |
| HROG19    | M   | 69  | GBM (IV)                   | L; temporoparietal       | † 15     |        |
| HROG20    | M   | 34  | Diffuse Astrocytoma (II)   | L; temporal              | 24       |        |
| HROG21    | M   | 44  | Secondary GBM (IV)         | R; parietal              | 21       | C      |
| HROG22    | M   | 66  | Relapsed GBM (IV)          | L; temporal              | † 4      |        |
| HROG23    | F   | 60  | Relapsed GBM (IV)          | L; parietal              | 20       |        |
| HROG24    | F   | 73  | GBM (IV)                   | L; occipital             | † 10     | C      |
| HROG25    | F   | 77  | Relapsed GBM (IV)          | L; temporal              | † 3      |        |
| HROG26    | M   | 63  | Relapsed Astrocytoma (II)  | R; parietal              | † 8      |        |
| HROG27    | M   | 76  | Meningioma (I)             | cerebrum                | 23       |        |
| HROG28    | F   | 76  | Meningioma (I)             | cerebrum                | ? 4      |        |
| HROG29    | M   | 39  | Diffuse Oligoastrocytoma (II) | cerebrum                | 19       |        |
| HROG30    | M   | 67  | Meningioma (I)             | frontal                 | ? 3      |        |
| HROG31    | F   | 59  | GBM (IV)                   | R; occipitotemporal      | 21       |        |
| HROG32    | F   | 76  | GBM (IV)                   | R; temporal              | 22       |        |
| HROG33    | F   | 46  | GBM (IV)                   | L; occipitotemporal      | † 13     | C, X   |
| HROG34    | F   | 69  | GBM (IV)                   | L; frontal               | † 5      |        |
| HROG35    | M   | 64  | Relapsed GBM (IV)          | R; occipital             | † 6      |        |
| HROG36    | F   | 80  | GBM (IV)                   | R; parietal              | † 5      | C      |
| HROG37    | F   | 20  | Pilocytic Astrocytoma (I)  | L; occipital             | ? 2      |        |
| HROG38    | F   | 49  | GBM (IV)                   | R; parietooccipital      | 19       | C      |
| HROG39    | F   | 59  | Meningioma (I)             | cerebrum                | 18       |        |
| HROG41    | M   | 71  | Secondary GBM (IV)         | L; frontal               | † 2      | C      |
| HROG42    | F   | 70  | GBM (IV)                   | L; frontal               | 16       |        |
| HROG43    | M   | 55  | Meningioma (I)             | L; frontal               | ? 8      |        |
| HROG44    | M   | 69  | Meningioma (I)             | L; frontal               | ? 8      |        |
| HROG45    | M   | 61  | relapsed Astrocytoma (II)  | L; parietal              | 13       |        |
| HROG46    | F   | 69  | GBM (IV)                   | R; parietotemporal       | 15       |        |
| HROG47    | M   | 59  | GBM (IV)                   | R; temporal              | † 16     |        |
| HROG48    | M   | 13  | Pilocytic Astrocytoma (I)  | L; occipital             | 13       |        |
| HROG49    | M   | 45  | Relapsed secondary GBM (IV) | R; parietooccipital      | ? 6      |        |
| HROG50    | F   | 33  | Diffuse Oligoastrocytoma (II) | L; frontal              | 14       |        |
| HROG52    | M   | 47  | GBM (IV)                   | L; temporobasal          | 13       | X      |
| HROG53    | F   | 50  | Anaplastic Astrocytoma (III) | cerebrum                | ? 4      |        |
| HROG54    | M   | 58  | GBM (IV)                   | R; parietal              | 8        |        |
| HROG55    | F   | 74  | GBM (IV)                   | R; parietal              | ? 1      |        |
| HROG56    | F   | 76  | GBM (IV)                   | trigonum                | ? 5      |        |
| HROG57    | F   | 60  | Relapsed GBM (IV)          | R; parietal              | 8        | C      |
| HROG58    | F   | 57  | GBM (IV)                   | R; frontal               | 7        | C      |
| HROG59    | M   | 60  | Relapsed GBM (IV)          | R; temporal              | † 8      | C, X   |
| HROG60    | M   | 51  | Relapsed GBM (IV)          | R; temporal              | ? 1      | C      |
| HROG61    | F   | 50  | diffuse Astrocytoma (II)   | L; frontal               | 6        | C      |
| HROG62    | M   | 71  | GBM (IV)                   | R; temporoparietal       | 4        | C      |
| HROG63    | M   | 48  | Relapsed GBM (IV)          | L; temporal              | 3        | C      |
| HROG64    | F   | 57  | GBM (IV)                   | R; temporal              | 1        |        |

The table summarizes patient information on sex (F=female; M=male), age at time point of resection in years, histological diagnosis including WHO grading in parentheses, tumor localization (L=left hemisphere; R=right hemisphere) and survival (†=patient died; bold=patients still alive on January 25th 2013; ?=no information available for > 6 months) in months after resection. Successful cell line establishment is indicated by a C, cell lines characterized in the following are bold and establishment of xenografts by an X. The X* indicates that a xenograft could be established but was lost subsequently.

Table 1: Patient and modeling data.
Mutations (TP53, IDH 1 and 2, KRAS, BRAF, PTEN)

Samples underwent analyses for the following loci: IDH 1 R132 (exon 4), IDH 2 R172 (exon 4), BRAF V600 (exon 15), KRAS G12, G13 (exon 2) and Q61 (exon 3), TP53 (exons 5 to 8) and full length PTEN (cDNA). The desired regions were amplified by PCR using specific primers (see Table 2). The PCR was performed using MiYaqHS polymerase (Bioline) according to the manufacturer’s recommendations. The PCR reaction was controlled by agarose gel electrophoresis and 15 µl of the products were purified using 3U of FAST AP Alkaline Phosphatase and 30U of Exonuclease I (Thermo Scientific, Schwerte, Germany) by incubation at 37°C for 15 min and subsequent heat inactivation at 85°C for 15 min.

One microliter of the PCR product was used as template for Sanger sequencing using BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Darmstadt, Germany) and the primers used for PCR according to the manufacturer’s protocol. The sequencing products were purified using the BigDyeXTerminator Purification kit (Applied Biosystems). The sequence was analyzed using the Applied Biosystems3500 genetic analyzer system and the SeqScape Software v2.7 (Applied Biosystems).

EGFR amplification

For determination of EGFR copy number, quantitative PCR was performed (primers see Table 2). 30 ng gDNA were used as template. The run was performed on a StepOneRealtime PCR system (Applied Biosystems) using Fast SYBR Green Mastermix (Applied Biosystems) with PBS, counted and 5 × 10⁵ cells were stained with 1 µg of the respective antibody or isotype control (see Table 3) for staining cell surface molecules. Cells were washed with PBS and resuspended in a final volume of 200 µl PBS. In case of unlabeled primary antibodies, excess antibody was washed out with PBS and respective secondary antibodies were added and final wash step was performed as above.

For the staining method with unlabeled primary antibodies (see Table 3), cells handled the same way with no primary antibody served as negative controls. All incubations were performed on ice for 30 min.

Cytokine secretion (ELISA)

Cells (5 × 10⁴ cells) were plated in 5 ml media per well in duplicates in 6-well culture plates and allowed to attach for 24 h. The media were

| Target     | Primer sequence forward | Reverse                |
|------------|-------------------------|------------------------|
| IDH 1 (Exon 4) | 5'-GCACGCGTCCTCCAGAGAAGGCC-3' | 5'-CACATTATTGCCCAACATAGC-3' |
| IDH 2 (Exon 4) | 5'-GCCCAACATTTGGCAGCCTACTA-3' | 5'-CAGGAAACAAGAGATTGGCTAGG-3' |
| BRAF (Exon 15) | 5'-TCTATAGTGGTCTGCTGTAGAGGA-3' | 5'-CTTCTCTAGTAACCTCGACGC-3' |
| KRAS (Exon 2) | 5'-GACGTAGCTGGTATTTTGAGTGTATTA-3' | 5'-TCAAGAATGGTCTGCTGACC-3' |
| KRAS (Exon 3) | 5'-CTTGGAGACGACAAACATGGTC-3' | 5'-TGCACAAAGAAGCGCCCTCCC-3' |
| TP53 (Exon 5) | 5'-GGTCTTTCCTCCGCGCTGTCGG-3' | 5'-GAACATAGTGAAGGAACTCAGA-3' |
| TP53 (Exon 6) | 5'-AGAGACAGACGAGCGCTGTT-3' | 5'-CACAATACCGAGAGGAAAGGC-3' |
| TP53 (Exon 7) | 5'-AAAAGGCGCTCCAAGCTTCG-3' | 5'-TGGACAAATCTCGTAGAGGCTG-3' |
| TP53 (Exon 8) | 5'-CGAGGTTCTGGTGGAGAGTAGA-3' | 5'-GAGGCAAGGAAAGGTGATAA-3' |
| PTEN (segment 1) | 5'-TTGCCATCTGAGAAGAAGGC-3' | 5'-GCTGGTGCTGGTATGGCCTG-3' |
| PTEN (segment 2) | 5'-ACGCCGCAAATTTAATGGCAG-3' | 5'-CGCCACTGACGACAGGAATTA-3' |
| PTEN (segment 3) | 5'-GTCATCAAGCGACAGCTGAGC-3' | 5'-CTGCACGGTACACTGCAGAAGAAGG-3' |
| PTEN (segment 4) | 5'-ACCCAGGACAGGAGAAACCT-3' | 5'-AAGGTCTCATTTCATTTATCAG-3' |
| EGFR | 5'-TCCCATAGTGATGCCTGCTCCTCACA-3' | 5'-CGGAAATGTCTGCTGACCTAAG-3' |
| LINE1 | 5'-TGCTCTTGAAATGCGTCCCAGAAG-3' | 5'-AAAGCGCGTCAATCATGG-3' |
| MGMT methylation | 5'-GCTGCTTGCTGCTGCTGTAAGT-3' | 5'-CACCTTTCGAAAAACGAAACAG-3' |
| Probe (MGMT) | 5'-6FAM-CGCAAACGATACGCACCGCGA-TMR-3' | 5'-AACGCGGCGTCAAAGACGAG-3' |
| MGMT expression | 5'-CCGGGACTTGGAGAGGTCC-3' | 5'-TCCCATGATCACACCTCTTCA-3' |
| COL2A1 | 5'-TCTAACATTATAAACCTCCACCACCAAC-3' | 5'-GGGGAAGATGGGTGAAGGGAATAT-3' |
| Probe (COL2A1) | 5'-6FAM-CTCTTGTCTCTTCTTGGAATCTTATCCTCACCCTCACCTTAA-3' | 5'-CGTACGCCTGCTTATCTCAGCAAA-3' |
| PTEN expression | 5'-ACGGAGACAGGAGAAACCT-3' | 5'-TGACGAGCTCTGATGCTGACAAAG-3' |
| TBP | 5'-TCGAGAGATTTCTCGGATGTT-3' | 5'-CACGAAATGTCTGCTGACCTAAG-3' |

The table lists all primers used in this study

Table 2: Primers.

Chromosomal instability

Chromosomal instability was assessed using the comparative genomic hybridization (CGH) based SNP Array 6.0 from Affymetrix (Cleveland, OH, USA) according to manufacturer’s instructions.

HLA typing

A 2-digit resolution typing of the following HLA loci was performed: HLA-A, -B and -C and HLA-DR and -DQ.

Flow cytometry

Cells were harvested by incubation with trypsin; the enzymatic reaction was stopped by adding cell culture media. Cells were washed with PBS, counted and 5 × 10⁵ cells were stained with 1 µg of the respective antibody or isotype control (see Table 3) for staining cell surface molecules. Cells were washed with PBS and resuspended in a final volume of 200 µl PBS. In case of unlabeled primary antibodies, excess antibody was washed out with PBS and respective secondary antibodies were added and final wash step was performed as above.

Similarly, 5 × 10⁵ cells were fixed with 2% Formafix and then treated with buffer P for 10 min to permeabilize the cell membrane for an intra-cellular staining. Cells were incubated with the antibody and washed with buffer P. After a second 10 min incubation period the respective secondary antibody was added in buffer P. Cells were washed and resuspended in 2% Formafix at a final volume of 200 µl.

For the staining method with unlabeled primary antibodies (see Table 3), cells handled the same way with no primary antibody served as negative controls. All incubations were performed on ice for 30 min.

Cytokine secretion (ELISA)

Cells (5 × 10⁴ cells) were plated in 5 ml media per well in duplicates in 6-well culture plates and allowed to attach for 24 h. The media were
replaced by fresh media or media not containing FCS (for TGF beta secretion). One ml samples of supernatant were collected 3 days and 5 and stored at -80°C. For detection of cytokine production samples were thawed on ice and 100 µl supernatant was used for each ELISA assay. IL-6 (matched pair; Immunotools, Friso, Germany), IL-8 (matched pair; Immunotools), TNF alpha (set pair; Immunotools), CEA (RayBio, Norcross, GA, USA) and TGF beta (Assaypro, St. Charles, MO, USA) ELISA assays were performed according to the manufacturer’s instructions.

### Drug response

Cells (5 x 10^4 cells) were plated in 150 µl media per well in triplicate in 96-well flat bottom culture plates and allowed to attach for 24 h. The following concentration ranges of drugs were tested (given are final concentrations in the experimental wells): (1) 500 µM-32 nM BCNU (Bristol-Myers Squibb, New York, USA), (2) 500 µM-32 nM CCNU (Lonustine, Sigma-Aldrich, St. Louis, MO, USA), (3) 1 mM-64 nM Cetuximab (Molekula, Mindel, Germany), (4) 30 µM-30 nM Cisplatin (Teva Gmbh, Uml, Germany), (5) 500 µM-125 nM Cytarabine (Cell Pharm Gmbh, Bad Vilbel, Germany), (6) 1 mM-244 nM Irinotecan (Pfizer, Berlin, Germany), (7) 1 mM-1 µM Methotrexate (Teva Gmbh), (8) 50 µM-3.2 nM Procarbazine (Natalan, Sigma-tau, Regensburg, Germany), (9) 30 µM-30 nM Rapamycin (Sirolimus, Pfizer), (10) 40 µM-10 nM Thalidomide (Sigma-Aldrich), (11) 2 mM-128 nM TMZ (Sigma-Aldrich), (12) 5 µM-320 PM Topotecan (GlaxoSmithKline, Munich, Germany), (13) 244 nM-300 PM Vinpircetine (Hexal, Holzkirchen, Germany) and the therapeutic antibodies 2.5 mg/ml-39 ng/ml Bevacizumab (Avastin, Roche, Basel, Switzerland) and 20 µg/ml-313 ng/ml Cetuximab (Erbitux, Bristol-Myers Squibb). Equal volumes DMSO (for cells treated with TMZ and BCNU) were added to cells serving as live control. Cells were incubated with the substances for 72 h, and media were replaced together with substances in the same concentrations as before. After another 72 h incubation period cells serving as dead control were incubated with 70% ethanol for 30 min and viability was assessed by using the viability dye calcein AM (ebiScience, Frankfurt, Germany) in a final concentration of 0.7 μM in fresh medium/PBS (2:1). Cells were incubated at 37°C in the dark for 20 min, fluorescence intensity was assessed using the microplate reader Infinite M200 (Tecan, Mennedorf, Switzerland) with 485 nm excitation, 535 nm emission and a constant gain of 160. Values were normalized (1=value live control; 0=value dead control).

### Molecular data

Molecular features of GBM such as the methylation status of the MGMT promoter, the amplification rate of EGFR, as well as mutation status of the genes IDH 1 and 2, TP53, KRAS, BRAF and PTEN were assessed in comparison to the original tumor material (Table 4). The methylation status of the MGMT promoter was consistent between original tumor and cell lines. Methylation of the promoter occurred in11/42 tumors and was maintained in the cell lines HROG02, HROG05, HROG13, HROG15 and HROG17. This coincided with no or only marginal cDNA expression (<0.001; Table 4). No methylation of the MGMT promoter was detectable in 27/42 tumors; for four samples the status could not be assessed; in two cases no snap frozen tumor tissue could be collected due to tumor size and in the two remaining cases no DNA could be isolated due to a high degree of necrosis. The tumors HROG24 and HROG36 were scored unmethylated; however cDNA expression analyses revealed marginal expression of MGMT cDNA for both cell lines (<0.001; Table 4). Further the cell lines HROG04, HROG06, HROG07, HROG10 and HROG38, which were also scored unmethylated, did express detectable levels of MGMT cDNA (Table 4).

All cell lines expressed detectable levels of PTEN cDNA; however, mutations in the gene were very frequently (8/13; 62%) detected (HROG04, HROG05, HROG06, HROG15, HROG17, HROG24, HROG36 and HROG38). No mutations in PTEN were detected in cell lines HROG02, HROG07, HROG10, and HROG13.

A genomic amplification of the EGFR was present in 22/42 (52%) tumors; 13 of these tumors had a high amplification (>10x). No amplification was detectable in 15/42 (36%) tumors and five samples could not be analyzed. In one case no DNA could be isolated in the first place and in the four remaining cases only little DNA could be isolated and this was not sufficient to perform all molecular analyses. The amplification rate of the EGFR differed in eight (HROG02, HROG04, HROG05, HROG06, HROG07, HROG10, HROG17 and HROG24) out of the twelve cases when comparing the status of the original tumor to the one of the cell line (Table 4). Loss of the genomic EGFR amplification is a frequently described phenomenon in literature and explained by extra-chromosomal EGFR amplification (in form of mini-chromosomes) which is gradually lost in cell culture due to absence of selective pressure [18-20].

Of note, all mutations of the original tumors were maintained in the cell lines except those affecting IDH 1. HROG02, HROG06, HROG15...
The table lists all antibodies used in this study for flow cytometry.

Table 3: Antibodies.

| Species   | Target | Clone | Label | Manufacturer                     |
|-----------|--------|-------|-------|----------------------------------|
| Mouse     | CD15   | MEM-158 | PE    | Immunotools (Friesoythe, Germany) |
| Mouse     | CD24   | SN3    | PE    | Immunotools                      |
| Mouse     | CD34   | -581-  | PE    | Immunotools                      |
| Mouse     | CD44   | MEM-85 | APC   | Immunotools                      |
| Mouse     | CD90   | AS02   | FITC  | Dianova (Hamburg, Germany)       |
| Mouse     | CD133  | AC133  | PE    | Miltenyi (BergischGladbach, Germany) |
| Mouse     | GFAP   | G5     | FITC  | eBioscience (Frankfurt, Germany) |
| Mouse     | Nestin | 10C2   | FITC  | eBioscience                      |
| Mouse     | S-100  | B32.1  | None  | Abcam (Cambridge, United Kingdom) |
| Mouse     | Vimentin | V9    | None  | Abcam                            |
| Goat      | Mouse  | polyclonal | PE    | DakoCytomation (Hamburg, Germany) |

This table summarizes molecular characteristics of tumors in comparison to the corresponding cell line. The molecular data was then used for sub-typing according to Verhaak et al. [7]. Listed are the methylation status of the MGMT promoter (M=methylated; U=unmethylated), the relative cDNA expression of the MGMT gene compared to the housekeeping gene TBP, the relative cDNA expression of the PTEN gene compared to the housekeeping gene TBP, the genomic amplification rate of the EGFR compared to the normal diploid status (1=2n) and detected mutations of the genes TP53, PTEN, IDH 1 and 2, KRAS and BRAF (wt=wild type, if no mutations were detected; mutations are indicated by the position with the wt amino acid in front and the amino acid resulting from the mutation behind or * in case of a stop codon; CN=copy number 1, when one copy of the gene was lost and 0 if both copies of the gene were lost; del=deletion of amino acids; spliced=alternatively spliced; +1=insertion of a base leading to a frame shift)

Table 4: Molecular characteristics.

| Sample ID | MGMT promoter status | cDNA expression | PTEN | EGFR [x fold] | Mutations | Molecular sub-classification |
|-----------|----------------------|-----------------|------|---------------|-----------|-----------------------------|
| HROG02    | tumor cell line      | M               | <0.001 | 3.68          | TP53 R248Q | Proneural                   |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | TP53 mut; 4q12 (PDGFRA) amplified |
| HROG04    | tumor cell line      | U               | 1.02  | 12.73         | PTEN W274L | Classical                    |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | EGFR amplified; 9p21.3 (CDKN2A) deleted |
| HROG05    | tumor cell line      | M               | <0.001 | 1.01          | 82        | Mesenchymal*                 |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | KRAS G12D PTEN P169S/del 212-229 |
| HROG06    | tumor cell line      | U               | 0.07  | 2.31          | 82        | Proneural                    |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | TP53 R273H/308* PTEN (+1 at 126) |
| HROG07    | tumor cell line      | U               | 0.34  | 14.92         | 12        |                              |
|           |                      |                 |       |               |           | Classical                    |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | EGFR amplified; 9p21.3 (CDKN2A) deleted |
| HROG10    | tumor cell line      | U               | 0.27  | 3.73          | 2         |                              |
|           |                      |                 |       |               |           | Proneural                    |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | 4q12 (PDGFRA) amplified      |
| HROG13    | tumor cell line      | M               | <0.001 | 2.88          | 1         |                              |
|           |                      |                 |       |               |           | Classical                    |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | chr.7 amplified; chr.10 lost; 9p21.3 (CDKN2A) deleted |
| HROG15    | cell line            | M               | <0.001 | 3.70          | 1         | Mesenchymal                  |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | TP53 R273H PTEN S170N         |
| HROG17    | tumor cell line      | M               | <0.001 | 0.55          | 4         | Mesenchymal*                 |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | PTEN R130* PTEN mutated      |
| HROG24    | tumor cell line      | U               | <0.001 | 2.21          | 43        | Proneural                    |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | TP53 R273C PTEN exon 3 del/spliced |
| HROG36    | tumor cell line      | U               | <0.001 | 3.72          | 1         | Mesenchymal*                 |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | MGMT CN=0 PTEN I5S            |
| HROG38    | tumor cell line      | U               | 0.23  | 0.02          | 1         | Mesenchymal*                 |
|           |                      |                 |       |               |           |                              |
|           |                      |                 |       |               |           | PTEN I224M/R234W             |

This table summarizes molecular characteristics of tumors in comparison to the corresponding cell line. The molecular data was then used for sub-typing according to Verhaak et al. [7]. Listed are the methylation status of the MGMT promoter (M=methylated; U=unmethylated), the relative cDNA expression of the MGMT gene compared to the housekeeping gene TBP, the relative cDNA expression of the PTEN gene compared to the housekeeping gene TBP, the genomic amplification rate of the EGFR compared to the normal diploid status (1=2n) and detected mutations of the genes TP53, PTEN, IDH 1 and 2, KRAS and BRAF (wt=wild type, if no mutations were detected; mutations are indicated by the position with the wt amino acid in front and the amino acid resulting from the mutation behind or * in case of a stop codon; CN=copy number 1, when one copy of the gene was lost and 0 if both copies of the gene were lost; del=deletion of amino acids; spliced=alternatively spliced; +1=insertion of a base leading to a frame shift)

and HROG24 show a mutation in the TP53 gene; HROG05 has a mutation in the KRAS gene. One mutation of BRAF was detected in the tumor HROG23 (no successful culture, data not shown). Mutations in the gene IDH 1 were present in tumors HROG21 (still in culture – but very slowly growing) and HROG41; the mutation however was not maintained in the cell line HROG41 (data not shown). The MGMT gene was completely deleted in HROG36 and only one allele was left in HROG24.

No mutations in the analyzed genes were detected in tumors and cell lines HROG07, HROG10 and HROG13.

Table 5: Moleculr characteristics.

CGH array

A variety of chromosomal abnormalities are described for GBM (see above). For a detailed analysis addressing this issue in the GBM cell line collection (except for HROG38), a genomic analysis with very high resolution taking advantage of the SNP Array 6.0 from Affymetrix was performed. All except one cell line (HROG07) showed almost complete loss of at least one copy of chromosome 10. Chromosome 1q was deleted in 4/11 (HROG02, HROG05, HROG24 and HROG36). The most frequent amplification was for chromosome 7; in 8/11 cases an amplification was present (HROG04, HROG05, HROG06, HROG13, HROG04 and HROG23).
HROG15, HROG17, HROG24 and HROG36). The long arm of chromosome 9 (9q) was amplified in cell lines HROG02, HROG04, HROG05, HROG07, HROG17 and HROG36. Merely cell line HROG13 had a deletion at 1p and HROG36 at 19q; no co-deletions of the loci were detected (for a detailed view see supplementary Figure 1).

**Molecular sub-typing**

According to the molecular data and pieces of information obtained by the CGH arrays, an attempt at sub-classifying the cell lines into the proneural, neural, mesenchymal and classical GBM types was undertaken (see Table 4). All but four cell lines could easily be assigned to one specific sub-type. The cell lines HROG05, HROG17, HROG36 and HROG38 could not be categorized definitely. Despite the fact that HROG05, HROG17 and HROG36 lacked the loss of 17q11.2 and for HROG38 no CGH data was available, they were assigned into the mesenchymal sub-type; basing on the fact that all four cell lines had mutated PTEN genes and since the mesenchymal sub-type is the most common one described for GBM cell lines [7].

All in all, 5/12 (42%) cell lines were categorized as/assigned to the mesenchymal sub-type, 4/12 (33%) were categorized as proneural and 3/12 (25%) as classical sub-type. None of the cell lines was classified as neuronal sub-type, which mainly is due to the lack of robust markers here for [7].

**Neuronal and cell surface marker expression**

The expression of neuronal markers such as GFAP, nestin, vimentin and S-100 as well as GBM (associated) cell surface and Brain Tumor Stem Cell (BTSC) markers was analyzed by flow cytometry (see Figure 2). In all cell lines general expression of neuronal markers was detectable (Figure 2A). A high level of general GBM (associated) markers was detectable. The degree of expressed BTSC markers varied from cell line to cell line, but a small positive population was always present (Figure 2B).

**Cytokine secretion**

The level of secreted cytokines with immunosuppressive and/or tumor relevant functions was assessed. All but one (HROG38) GBM cell line secreted high levels of IL-8. High secretion of IL-6 was detectable in 6/12 (50%) cell lines: HROG06, HROG10, HROG15, HROG17, HROG24 and HROG36. Little IL-6 was present in the supernatant of HROG05; and cell lines HROG02, HROG04, HROG07, HROG13 and HROG38 secreted no IL-6. Merely, the cell line HROG04 secreted some TGF beta (see Table 5). None of the cell lines secreted CEA or TNF alpha (data not shown).

**HLA typing and tumor (associated) antigens**

For future development of immunotherapeutic strategies a two-digits encompassing HLA typing was performed on the cell lines (see Table 6). The analyses revealed that 10/12 (83%) cell lines were HLA-A2 positive. The cell lines were further analyzed for the expression of tumor associated antigens (TAA) such as CEA, IL-13 receptor alpha (IL-13Ra), TGF beta and HIP-1; all in the discussion as being relevant in GBM tumors [21-25].

Rather high levels of CEA were detectable in all cell lines. In contrast, the degree of GBM TAA varied less from cell line to cell line; generally only few cells stained positive for GBM TAA, yet a small positive population was always present (Figure 3).
The amount of cytokines secreted after 72 hours (value before the slash) and 120 hours (value after the slash) of cell culture are listed in Table 5: Cytokine secretion.

Table 6: HLA typing.

Calculated IC₅₀ values (from three independent assessments in triplicates) for 144 hour incubation periods with the therapeutic agents are provided for all cell lines in Table 7a: Drug sensitivity for conventional chemotherapeutics (IC₅₀ values).

Calculated IC₅₀ values (after three independent assessments in triplicates) for 144 hour incubation with the therapeutic agents are provided for all cell lines Table 7b: Drug sensitivity for novel and targeted therapeutics (IC₅₀ values).

Drug response

Finally, response of the GBM cell lines to increasing doses of therapeutic agents was assessed (IC₅₀ values; Table 7). Sensitivity to an agent varied between cell lines and sensitivity of a cell line to various agents differed as well. For CCNU, Cisplatin, Cytarabine and Topotecan a correlation with the methylation status of the MGMT promoter or with cDNA expression levels were found. *In vitro* sensitivity was significantly higher in hypermethylated (cDNA expression<0.001) cell lines; with p=0.033 for CCNU, p=0.002 for Cisplatin, p=0.016 for Cytarabine and p=0.024 for Topotecan. In the case of the remaining alkylating substances BCNU, Procarbazine and TMZ, however, no correlation of sensitivity towards the agents and the methylation status were found.
methylated Glioblastoma Multiforme Model Establishment. J Cancer Sci Ther 6: 177-187. doi:10.4172/1948-5956.1000269

Discussion

In the present study we aimed at generating a collection of GBM models reflecting the clinical appearance of GBM cases. Prime focus was characterizing the GBM models in detail for subsequent translational approaches such as response prediction and therapy development.

Final establishment of permanent growing cell lines was more successful than in vivo engraftment; contrary to success rates for colorectal carcinomas in our group [26] but very much in line with data from the literature [27].

The HROG cell line establishment rates are for the most part superior to what is described in literature with success rates ranging from 3% for pediatric brain tumors [28] over 10% [29] to 21% [30] for adult GBM tumors. However, most authors do not comment on any statistics at all. The most stably outgrowing and subsequently characterized twelve cell lines of our collection included eight newly diagnosed and four relapsed tumors, meaning successful establishment of an immortal and stably growing cell line in 29% of cases for both newly diagnosed and relapsed tumors. There are another 13 cultures also stably outgrowing but not yet fully characterized mainly due to very high doubling times (>100 hours). When possible, characterization is continuously ongoing (e.g. HROG33, HROG41, HROG59 and HROG63).

On the pro side of in vitro models are the fast and easily feasible method and high success rates [27] thus cell cultures provide a good model for a first drug screen on response and resistance development. Subsequent testing and verification may then more selectively be performed using in vivo models – preferably established in parallel to the cell lines.

One major drawback of tumor models in general is a trend towards genetic drift (in comparison to the original tumor material) [31]. Therefore, the GBM models generated in this work were compared to the primary GBM tumor tissue presented to the pathologist for routine diagnosis. High preservation of the primary GBM tumor's molecular features was achieved in the models. The only exceptions observed concerned the mutation status of the IDH 1 gene, which is not maintained in the in vivo models, and the genomic amplification of the EGFR which is lost during standard in vivo culturing processes. Both phenomena are well described in literature [18-20].

The extensive molecular pathological analyses are not only required for detailed diagnosis but also have clinical relevance. In breast carcinoma patients the HER2/neu receptor is relevant for therapy with the monoclonal antibody Trastuzumab; only patients with an amplification of the HER2/neu receptor profit of this therapy [32]. For GBM patients not responding to the first line therapy (radio-chemotherapy with TMZ) a variety of alternatives including targeted therapeutics are available. Patients with amplification of EGFR may receive monoclonal antibodies directed against this receptor. However, effectiveness of these antibodies seems to be restricted to the expression of the wild type form of the receptor [33]. Bevacizumab is an antibody directed against the growth factor VEGF and thus could inhibit tumor vascularization and (neo-) angiogenesis [34].

Many recent studies, i.e. clinical testing of targeted therapeutics, have not led to the expected results but rather fell short of the high expectations [35]. This is to a big part attributable to the very heterogeneous nature of GBM tumors [35] and thus comes as no big surprise. Apparently, if the great heterogeneity is not adequately considered when recruiting for clinical studies, possible beneficial effects for individual GBM sub-types may be undetected. In line with this argumentation is the initiative to sub-classify GBM tumors into the categories proneural, neural, mesenchymal and classical by designating specific molecular characteristics to these sub-groups [7]. The gene signature described by Duarte and coworkers [13] might have strong implications both for better prediction models for survival and improved understanding of the underlying subtype-specific molecular mechanisms for GBM tumor progression and treatment response.

A key feature of cancer development is the progressive accumulation of genomic alterations resulting in the loss of tumor suppressor functions, the activation of oncogenes and the generation of fusion genes with oncogenic potential [36]. Such complex structural and numerical alterations in the genome leading to changes in the DNA copy number are characteristic also of GBM tumors [37].

Beside defining and uncovering tumor initiating, propagating and metastasizing processes, and identifying new (molecular) target structures, high throughput screening of drugs is one main field of tumor model usage; especially cell lines for the latter. Cell cultures are broadly used since cell lines are easy to handle, and manageable in high quantities; they represent a relatively low-cost approach and are ethically preferable to methods utilizing experimental animals [38]. Thus they have somewhat become the pharmaceutical industries favorite "pet". However, one big obstacle remains. Cell lines established in the 1970s and 1980s, when there was a big hype for GBM cell lines [27] have been passed very frequently and mostly even uncounted times and certainly have “acquired” culturing artifacts. Many changes over long term in vitro culturing are well described. Extensive in vitro passaging may lead to a hypermethylated phenotype. In this respect, Danam and colleagues demonstrated that with increasing cell culture passage, methylation progressively increased and revealed a concomitant trend to a completely MGMT-silenced phenotype [39]. Acquisition of “new” mutations and chromosomal aberrations are further described for highly passaged cell lines [27]. These (very) long term cultures have little in common with the original situation in the patients and thus only have limited model capability and drug testing potential.

The gold standard chemotherapeutic agent for GBM tumors is since 2005 TMZ [40,41]. All novel therapeutics must measure up to it and prove significant benefit for GBM patients or lower toxicity towards normal tissue, i.e. have fewer side effects. We assessed responsiveness of the patient-derived low passage GBM cell line collection towards a broad range of chemotherapeutics. The sensitivity to CCNU, Cisplatin, Cytarabine and Topotecan correlated with the methylation status of the MGMT promoter or MGMT cDNA expression and was significantly
higher in hypermethylated cell lines. No correlation of the methylation status of the MGMT promoter could be detected for the agents BCNU, TMZ and Procarbazine. This is somewhat in contrast to the positive correlation described for methylated MGMT promoter and response to alkylating agents [3]. However, the presence of MGMT cDNA tended to correlate with a better response in methylated cell lines. This emphasizes the value of these patient-derived low passage cell lines since detailed characterization revealed for example a deletion of the entire MGMT sequence in HROG36 which was scored unmethylated but did not even harbor the sequence. The same holds true for HROG24 with only one copy of the MGMT gene. Methylation scoring for such cases may have to be reconsidered. Responses to Irinotecan and Topotecan tended to be strongest for cell lines with mutated TP53. This finding goes well with the fact that GBM cells treated with the DNA topoisomerase inhibitor SN-38 only underwent cell cycle arrest and even re-proliferated after withdrawal of the inhibitor in a wild type TP53 setting, whereas in cells with mutations in the tumor suppressor, treatment caused apoptosis [42]. Thalidomide, initially applied as a sedative, has proven anti-cancer efficacy [43,44]. Three cell lines: HROG05, HROG17 and HROG36 were highly responsive to the agent. Currently Lenalidomide (derived from Thalidomide; CC-5013) [45] only underwent cell cycle arrest and even re-proliferated after withdrawal of the inhibitor in a wild type TP53 setting, whereas in cells with mutations in the tumor suppressor, treatment caused apoptosis [42].

Acknowledgements

The cancer stem cell hypothesis was initiated in the 1990s by Dick and colleagues [45]. Their report on Leukemia initiating cells became the paradigm for later studies proposing cancer stem cells to be at the top of a hierarchical pyramid [46]. A number of strategies has been developed and tested to treat GBM tumors by specifically targeting brain tumor stem cells; e.g. by miRNA [47], CD133 specific antibodies [48] or by vaccination with brain tumor stem cell loaded dendritic cells [49].

In summary, our novel patient-derived (ultra-) low passage cell lines and matched xenografts represent model systems with ideal features for response and resistance prediction, and, since they are molecularly and clinically well characterized, will be essential tools for the next steps towards truly individualized therapy.

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