

EGF signalling and rapamycin-mediated mTOR inhibition in glioblastoma multiforme evaluated by phospho-specific flow cytometry

Isabelle Cornez · Mrinal Joel · Kjetil Taskén ·
Iver A. Langmoen · Joel C. Glover · Torunn Berge

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Abstract Development of novel patient stratification tools for cancer is a challenge that require advanced molecular screening and a detailed understanding of tumour signalling networks. Here, we apply phospho-specific flow cytometry for signal profiling of primary glioblastoma tumours after preservation of single-cell phosphorylation status as a strategy for evaluation of tumour signalling potential and assessment of rapamycin-mediated mTOR inhibition. The method has already enhanced insight into cancers and disorders of the immune system, and our study demonstrate a great potential to improve the understanding of aberrant signalling in glioblastoma and other solid tumours.

Keywords EGF signalling · Glioblastoma multiforme · Phosphoflow cytometry · Rapamycin

Isabelle Cornez and Mrinal Joel contributed equally to the study.

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I. Cornez · K. Taskén · T. Berge (✉)
The Biotechnology Centre of Oslo and Centre for Molecular
Medicine Norway, Nordic EMBL Partnership, University of
Oslo, Gaustadalléen 21, 0349 Oslo, Norway
e-mail: torunn.berge@biotek.uio.no

Present Address:

I. Cornez
Biomedizinisches Zentrum (BMZ), Medizinische Klinik III,
53127 Bonn, Germany

M. Joel · I. A. Langmoen
Vilhelm Magnus Laboratory for Neurosurgical Research,
Institute for Surgical Research, Oslo University Hospital,
Oslo, Norway

Introduction

Glioblastoma multiforme is a highly invasive malignant tumour of the central nervous system associated with a particularly poor prognosis (median survival 9–15 months) due to the aggressive nature of the cancer and a lack of efficient treatment options. The high degree of molecular and cellular heterogeneity within these tumours makes them difficult to treat, and the current treatment options consisting of surgery followed by radiation and chemotherapy are only palliative [1]. Primary glioblastoma commonly presents genetic alterations such as amplification of the epidermal growth factor receptor gene, *EGFR*, mutations or loss of the phosphatase and tensin homolog gene, *PTEN*, as well as mutations of the phosphatidylinositol 3-kinase *PIK3CA* gene [2–5]. *EGFR* amplification is also frequently accompanied by gene rearrangements resulting in receptor variants including the constitutively active EGFRvIII, a truncated receptor lacking parts of the extracellular domain and thus insensitive to EGF ligand binding [6].

M. Joel · J. C. Glover
Department of Physiology, Institute of Basic Medical Sciences,
University of Oslo, Oslo, Norway

M. Joel · I. A. Langmoen · J. C. Glover
Norwegian Center for Stem Cell Research, Oslo, Norway

K. Taskén
Department of Infectious Diseases, Oslo University Hospital,
Oslo, Norway

I. A. Langmoen
Department of Neurosurgery, Oslo University Hospital,
Oslo, Norway

EGFR-driven activation has been subjected to rigorous investigations in cancer biology due to the regulatory role of its associated downstream pathways (Ras/Raf/MAPK and PI3K/Akt) in the tight control of proliferation, survival and growth responses [7]. Inhibitors of EGFR and its related signal mediators are currently in clinical use for treatment of lung, colon and breast cancer [8, 9]. So far, these drugs have shown little effect in patients with glioblastoma, however, targeting aberrant regulation of effector molecules in these signalling pathways still remains an important strategy for developing a treatment for this cancer.

Inhibition of mammalian target of rapamycin (mTOR), a downstream effector of the PI3K/Akt pathway, by compounds such as temsirolimus (CCI-779), everolimus or rapamycin has been suggested as a potential therapy for glioblastoma, based on *in vitro* data showing reduced proliferation particularly of cells lacking the tumour suppressor PTEN [10–13]. PTEN is inactivated in ~50 % of all high-grade gliomas, either by deletion, mutation or epigenetic alteration [3, 14], and PTEN loss has been suggested to sensitize the tumour cells to mTOR inhibition [14–16]. PTEN functions as a lipid phosphatase by dephosphorylating PIP₃ to PIP₂, thereby down-regulating EGF-induced signalling by directly reversing the process catalyzed by activated PI3K. As a downstream effector, mTOR links growth factor receptor-activated signals with protein translation, proliferation and survival. However, mTOR is also part of a feedback complex that maintains full activation of Akt at the cell membrane, illustrating the complex role of mTOR in signal regulation [14].

Recent advances in molecular profiling technologies, such as genetic and metabolic screening, have opened for disease treatment strategies based on a more individualized approach by providing molecular assays to aid targeting of individual patient disease profiles. Such tailored therapies or ‘personalized medicine’ have already been successfully applied for immune-related diseases and certain cancers, improving both diagnosis and risk stratification [17]. Whereas several parameters utilized to phenotype tumour samples have a static nature, evaluation of dynamic events is key for monitoring the regulation of intracellular signalling in cancer cells and evaluating specificity and efficacy of drugs directed against particular targets in patient samples. In this report, we introduce phospho-specific flow cytometry (hereafter: phosphoflow) as a new molecular tool for evaluation of intracellular signalling in tumour samples from glioblastoma patients by visualizing changes in phospho-epitopes following growth factor-induced stimulation before and after treatment with the mTOR-inhibitor rapamycin. Correlating these signalling profiles with functional analyses, clinical parameters and biochemical differences may provide sufficient conditions as

companion diagnostics to assist in defining a treatment strategy that will improve the current outcome for this cancer.

Materials and methods

Cell culture

The U87MG cell line was acquired from ATCC. Tumour biopsies were obtained from consenting glioblastoma multiforme patients as approved by the Norwegian National Committee for Medical Research Ethics. Further treatment of biopsies and cell culture are described in Supplementary Materials.

Reagents for phosphoflow cytometry

Alexa647-conjugated antibodies for pAkt (pS473) and pS6 ribosomal protein (pS235/pS236) were purchased from Cell Signalling Technology. The unconjugated antibody for pERK1/2 (pT202/pY204) was from Sigma Aldrich, and Alexa647-coupled goat anti-mouse IgG and IgGκ antibodies were from Life Technologies and BD Biosciences, respectively. Pacific Blue™ succinimidyl ester (Life Technologies) was used for fluorescent bar coding.

Cell sample preparation and fluorescent bar coding

$1-4 \times 10^6$ U87MG or primary tumour cells were maintained over night in medium depleted of serum or EGF/FGF, respectively. When specified, cells were first treated for 7 days with the mTOR inhibitor rapamycin at a concentration of 1 nM (Calbiochem) in their respective cell culture medium. Stimulation was performed by exposure to 10 ng/ml EGF (Sigma Aldrich) for the indicated times. After stimulation, cells were put on ice and culture medium removed immediately by suction (U87MG) or after 5 min centrifugation ($300 \times g$) at 4 °C (tumour cultures). Next, cell suspensions were prepared followed by one-dimensional bar coding of the time points using Pacific Blue™ ester as described by [18] and in Supplementary Materials.

Immunostaining and phosphoflow cytometry

Bar coded cells were rehydrated and washed twice in flow washing solution, aliquoted and incubated with the different phospho-specific antibodies for 30 min at room temperature, followed by staining with secondary Alexa647-coupled antibodies (30 min at room temperature) when required. Next, the cells were washed ($1 \times$) with flow washing solution and re-suspended for flow cytometry using the same solution. Signal responses were analyzed

using a BD FACSCanto™ II (4-2-2) cytometer equipped with 405, 488 and 633 nm lasers. For each sample, at least 2,000 events from each cell population were recorded. Cytobank (<http://cytobank.org>) was used for further analysis and data visualization.

Cell proliferation

Proliferation was tested by XTT assay (Roche Diagnostics) using exponentially growing cells plated at a density of 1×10^4 cells per well in a 96-well plate. Five wells were measured for each sample at 490 nm using a plate reader (Victor™ X5, Perkin Elmer 2030) as described by the manufacturer.

Sphere formation assay

Glioblastoma tumour-spheres were dissociated to single-cell suspensions and 500 cells per well were plated in ultra-low attachment 96-well plates (Sarstedt). Sphere formation in ten wells per sample was evaluated 10 days after culture (with or without 7 days rapamycin treatment included). The number and cross-sectional diameter of spheres were measured using GelCount™ software (Oxford Optronix).

Statistics

Statistical significance was tested using Student's *t* test in Excel, Microsoft Office 2007.

cDNA libraries and DNA sequencing

Tumour cell mRNA was isolated using the Dynabeads mRNA direct™ kit (Life Technologies) according to the manufacturer's protocol. cDNA synthesis was performed

using RevertAid™ reverse transcriptase (Fermentas/Thermo Scientific), and PTEN was amplified by PCR using the primers ATG ACA GCC ATC ATC AAA GAG AT (forward) and TCA GAC TTT TGT AAT TTG TGT ATG CTG (reverse). PCR products were inserted into a vector using the Zero Blunt® TOPO® PCR cloning kit (Life Technologies) as described by the manufacturer and inserts were sequenced by GATC Biotech (GATC Biotech).

Results

Molecular characterization of glioblastoma patient samples

Tumour samples from six patients with primary glioblastoma multiforme were collected with patient consent (see also Supplementary Material). The sample set was complemented by the glioma cell line U87MG and the expression of selected molecular features was evaluated by Western blotting (Fig. 1a). U87MG, reported as lacking expression of PTEN and having low levels of wild-type EGFR [19], was used as a reference. EGFR amplification was detected in tumours T2, T5 and T6; the latter two also expressed the truncated receptor form EGFRvIII. By contrast, tumours T1 and T3 both expressed less EGFR than U87MG, and EGFR was not detectable at this exposure level in tumour T4. The tumour suppressor PTEN was present in tumours T1–T3 and T6 but absent from tumours T4 and T5. We also analyzed expression of the tumour suppressor p53, which was found expressed at a similar level in all samples (*data not shown*).

To evaluate EGFR-mediated signal flux in the tumour cells, we set up an assay monitoring the phosphorylation status of key effector molecules belonging to the main EGFR-associated pathways (Fig 1b). Activation of the Ras/

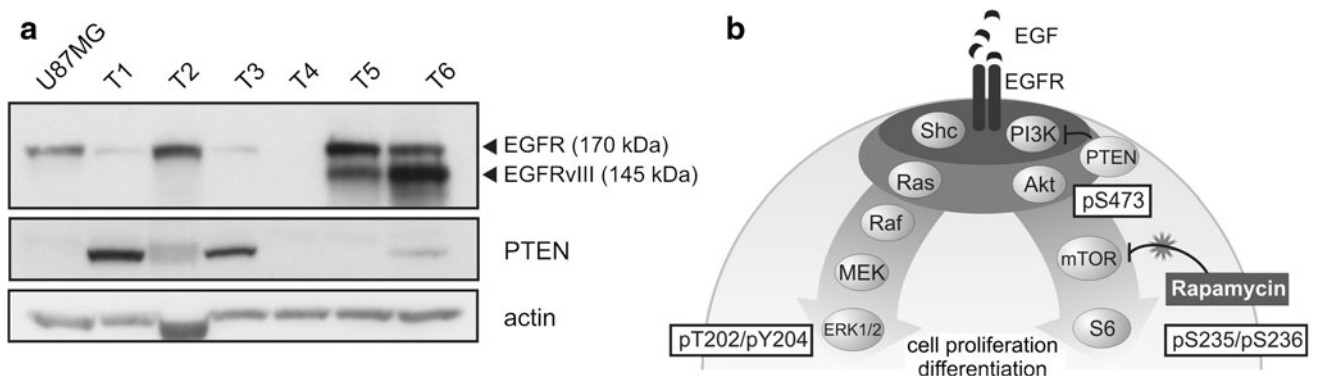


Fig. 1 Characterization of patient samples and molecular targets for EGFR signal profiling **a** U87MG and six primary glioblastoma samples were analyzed for EGFR and PTEN content by Western blotting using actin as a loading control. **b** Activation of glioblastoma

cell samples by EGF stimulation was assayed through selected signalling molecules downstream of the EGFR, belonging to the Ras/Raf (pERK1/2) and the PI3K/Akt (pAkt and pS6) pathways, respectively

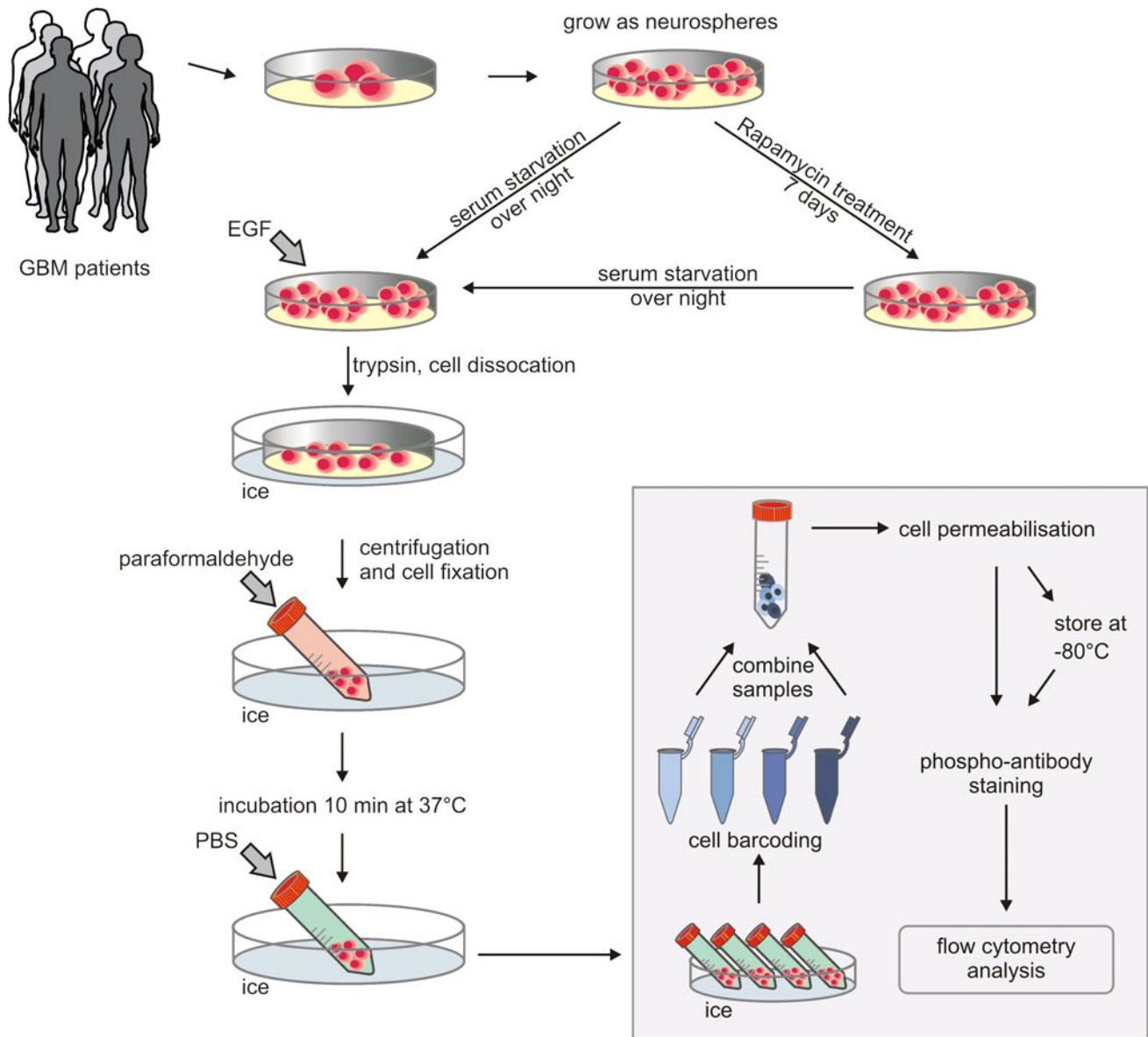


Fig. 2 Processing of solid tumour samples for phosphoflow cytometry. Cells from glioblastoma biopsies were cultured as tumour-spheres in appropriate culture medium. After 7–10 passages, the tumour-spheres were treated with rapamycin for 7 days or left untreated, then starved of growth factors overnight and exposed to EGF for different durations. Single-cell suspensions were prepared by ice-cold trypsination followed by immediate fixation after

dissociation to preserve protein phosphorylation and halt signalling. Fluorescent cell bar coding preformed through staining with set concentrations of Pacific Blue provided each time point with a unique identifier. This allowed subsequent pooling of the samples prior to staining with phospho-specific antibodies and multiparameter analysis by phosphoflow cytometry

Raf pathway was assessed by pERK1/2 (pT202/pY204), whereas pAkt (pS473) and pS6 (pS235/pS236) were used to monitor signal flux via the PI3K/Akt pathway. S6 ribosomal protein is a direct target of S6 kinase 1 downstream of mTOR and commonly serves as readout for mTOR activity and efficacy of mTOR inhibition [20]. The inclusion of pAkt in the analysis allowed us to examine the potential of mTOR as an upstream regulator of Akt activity [21]. Time course for the experiments was decided after

Western blot analysis performed to verify effect of overnight starvation and subsequent EGF activation of the cell samples (see Supplementary Material).

Flow cytometry-based analysis of signalling in adherent cells and solid tumours

Quantitative analysis of intracellular signal cascades by phosphoflow cytometry is well established in normal and

diseased immune cells as well as in equivalent suspension cell lines. Recent advances have made screening at a significantly higher throughput possible through the introduction of sample multiplexing by fluorescent cell bar coding (FCB) [18, 22]. By establishing a protocol including FCB to provide conditions that preserve intracellular phospho-epitopes in adherent or non-suspension cell populations, we applied the technology to profile signalling in glioblastoma samples (Fig. 2). Briefly, our protocol involved splitting cultured tumour-spheres for EGF stimulation, followed by cold-trypsin at given time points to arrest signal processes and provide single-cell suspensions. The ice-cold cell suspensions were subsequently fixed, bar coded for individual time points through staining with set concentrations of Pacific Blue dye, followed by sample pooling for permeabilisation and phospho-antibody staining. Consequently, samples were treated with identical antibody panels; minimizing both the amount of antibodies used and variation in staining intensities (see also Supplementary Material).

Phosphoflow evaluation of signal heterogeneity in glioblastoma multiforme

U87MG and the six glioblastoma samples were serum starved of serum or growth factors overnight, respectively, followed by exposure to 10 ng/ml EGF for 0, 7, 15 or 30 min. The samples were subsequently bar coded to identify these time points and stained for pERK, pAkt and pS6 for phosphoflow cytometry. Data were expressed as the arcsinh-transformed population median fluorescence intensity (MFI), and responses in each sample evaluated by calculating the signal change relative to unstimulated cells. For each experimental condition, we created signal heat maps by colour coding these responses so that small changes were represented by black while high or low activity is represented by pale yellow or dark blue, respectively (Fig. 3a).

In the primary tumour populations, significant responses were elicited by EGF in T1–T5 for all assayed effector molecules, whereas sample T6 was not activated by EGF. The lack of EGF-response in T6 may be explained by the high expression of truncated receptor EGFRvIII in this tumour. A high pS6 signature was seen in T1–T3 (arcsinh MFI > 2 eq. ~10-fold change); this response did not correlate with high pAkt signalling and considerable signal heterogeneity was found between the tumour samples. Exposure of the U87MG cell line to EGF elicited only a slight increase in phosphorylation at the later time points. Evaluation of basal activation levels revealed higher basal responses in U87MG than in any of the tumour samples for all readouts (Fig. 3b). Plotting the pS6 response data as histograms confirmed this (Fig. 3c), showing U87MG to

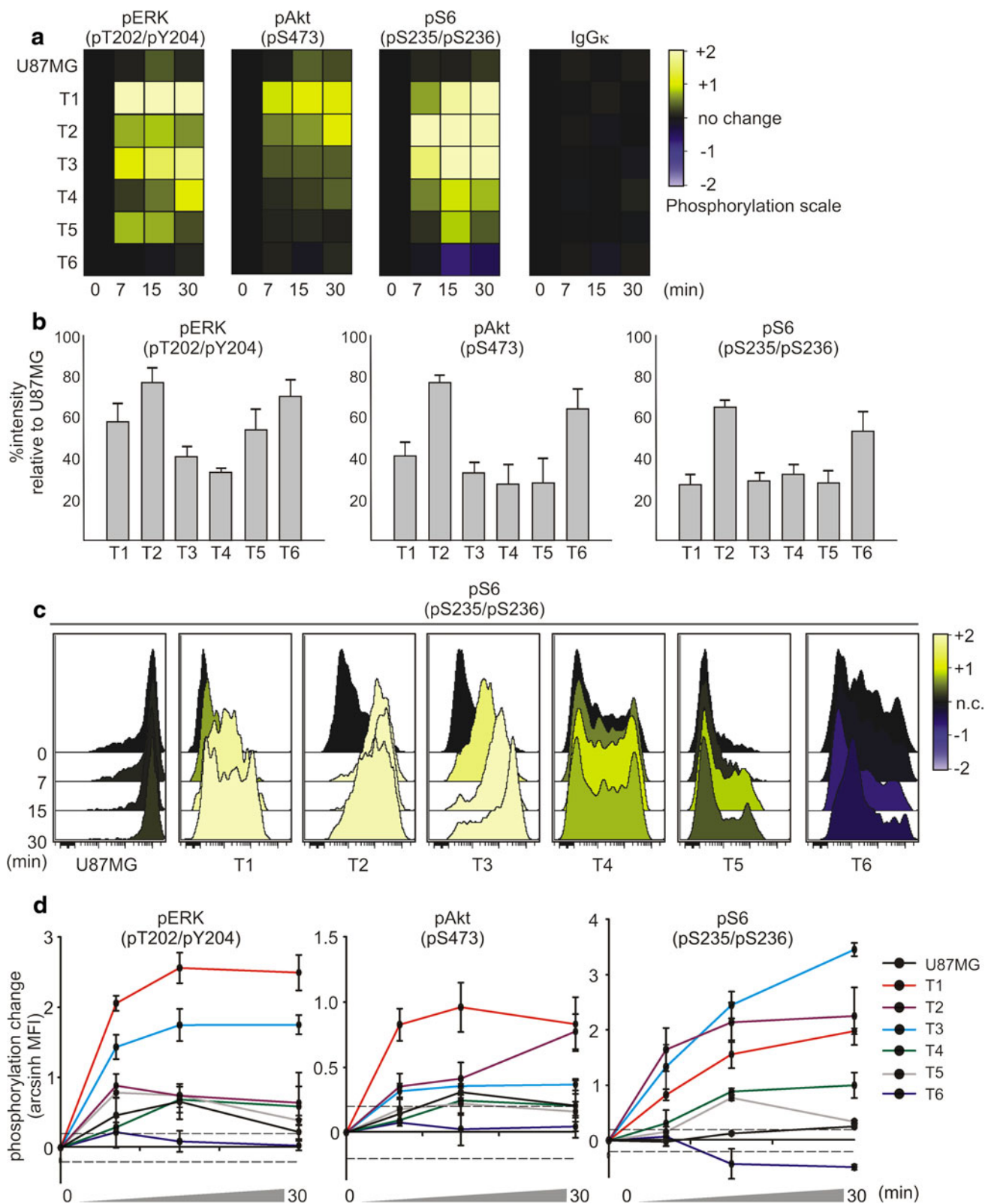
Fig. 3 Signal profiling of pERK, pAkt and pS6 by phosphoflow reveals EGF responsiveness in glioblastoma tumours **a** U87MG and patient samples (T1–T6) were exposed to EGF for 0, 7, 15 and 30 min and ERK1/2, Akt and S6 phosphorylation assessed by phosphoflow cytometry. Changes in protein phosphorylation relative to untreated cells were determined for each sample and represented in heat maps (no change/n.c. = black). IgGκ was included as a control for background fluorescence. Representative data from one experiment are shown ($n = 3$). **b** Basal activation of the tumour cultures evaluated relative to the MFI signal of U87MG expressed in percentage \pm s.e.m, $n = 3$. **c** Heterogeneity of EGF-mediated responses in the tumour samples was revealed by histograms showing the full distribution of pS6 signals. The histograms have been stacked to show dynamic changes following exposure to EGF using colour coding as described for the heat maps. Representative histograms are shown ($n = 3$). **d** Plotting the change in phosphorylation signal (average arcsinh MFI \pm s.e.m, $n = 3$) for each sample against time demonstrated the reproducibility of the method and identified high responders to EGF stimulation. The dashed line indicates background fluorescence representing signal range detected for control (IgGκ) staining

have a tight, homogenous population response, changing only slightly over the course of stimulation. In contrast, histograms of the tumour samples were substantially broader with heterogeneous features and a mixture of high and low responders giving rise to response peaks and/or shoulders.

Reproducibility of the method was tested by plotting the average MFI change (arcsinh scale) against time using data from cells grown at different passages (7–10) (Fig. 3d). This showed that tumours T1–T3 were consistently and significantly more responsive to EGF exposure than T4–T6 and U87MG. Within the high responding subset, T1 and T3 signalled most strongly at the pERK1/2 level, whereas T1 and T2 responded more strongly as measured by pAkt at 30 min. Based on these phospho-signatures, T1, T2 and T3 were selected for testing of sensitivity to rapamycin-mediated inhibition of mTOR. As indicated previously, T1–T3 were all PTEN-positive as tested by Western blotting (Fig. 1a). Sequencing revealed that T1 and T3 both contained intact PTEN sequences, whereas the PTEN sequence of T2 contained a single point mutation (C517T) resulting in an arginine to cytosine switch. This residue is located within the phosphatase domain, and thus could negatively affect functionality of the tumour suppressor.

Analysis of rapamycin-mediated mTOR inhibition in glioblastoma tumour cells

Following the workflow described in Fig. 2, T1–T3 and U87MG cells were treated with rapamycin for 7 days at a concentration of 1 nM, equivalent to the intratumoural concentration achieved in a phase I trial involving glioblastoma patients [15]. Analysis of EGF-responses in the treated samples (Fig. 4a, compare “+” column to “+R” column) showed that pERK1/2 was unaffected by



rapamycin in the tumour cells but increased slightly in U87MG cells. An increase in Akt phosphorylation occurred only in T1, a heterogeneity reminiscent of what has

been observed in a clinical evaluation of rapamycin, where ~50 % of enrolled glioblastoma patients experienced Akt activation and shorter time-to-progression as a negative

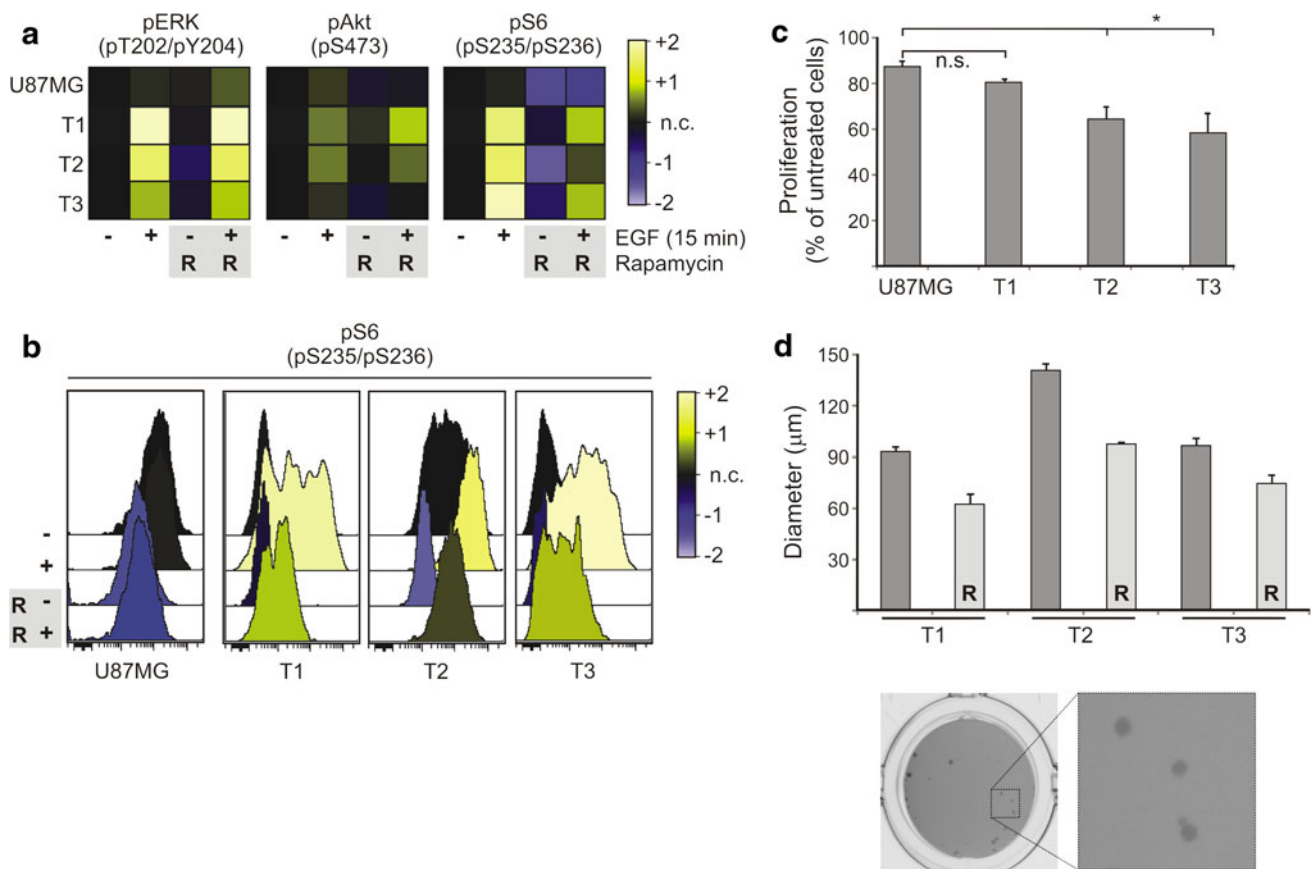


Fig. 4 Rapamycin-mediated mTOR inhibition in glioblastoma multiforme **a** U87MG and tumour samples identified by high pS6 signalling (T1–T3) were treated for 7 days with rapamycin at 1 nM (R). The cells were then starved overnight and stimulated by exposure to EGF for 15 min (+). Untreated cells were included as controls with unstimulated cells (–) used as a reference. The heat maps show data from one representative experiment ($n = 3$). **b** Phosphorylation of S6 after mTOR inhibition by rapamycin (R), analyzed by histograms to

show the full distribution of phosphorylated species for each cell sample. Representative data are shown ($n = 3$). **c** Cell proliferation, tested by XTT assays, shown as % proliferation in rapamycin-treated cell relative to untreated cells (mean \pm standard deviation, $n = 3$, 5 individual measurements per experiment). **d** Rapamycin treatment (R) contributed to a size reduction of the tumour-spheres, illustrated by a reduction in the average cross-sectional diameter (μm) \pm standard deviations ($n = 3$, 10 individual experiments)

side effect of the inhibition [15]. A significant reduction of S6 phosphorylation was detected in all samples, both during EGF-stimulation and at basal levels, indicating mTOR inhibition in both cases. Overall, rapamycin treatment reduced the heterogeneity of the pS6 response, seen as decreased width in histograms (Fig. 4b). The greater sensitivity to rapamycin treatment of tumour cells relative to U87MG cells was confirmed in proliferation assays, which showed a significantly greater reduction in T2 and T3 than in U87MG ($P < 0.05$) (Fig. 4c). However, despite a significant pS6 reduction following rapamycin exposure, proliferation in T1 was not significantly reduced relative to U87MG. This poor response may be explained by the parallel pAkt activation in the treated T1 cells. Overall, drug treatment resulted in reduced size of the tumour-spheres in vitro, measured as cross-sectional diameter (Fig. 4d).

Discussion

Despite several promising preclinical studies involving targeted agents, advances in treatment of glioblastoma multiforme have only been incremental [23]. Individual, specific therapies such as mTOR and EGFR kinase inhibitors have been evaluated in clinical trials but have not produced the desired consistent efficacy, in part due to variations in drug uptake and difficulty in administration across the blood–brain barrier. Furthermore, the heterogeneous nature, complex molecular features and high degree of redundancy in signalling networks of each tumour create difficulties both in diagnosis and for definition of good patient cohorts [19, 24].

Further progress in the search for efficient therapies for glioblastoma is being sought through research on molecular targets within overly active signal pathways such as the

PI3K/Akt pathway. To gain momentum, this approach will require improved screening technologies that provide detailed assessment of dynamic features of signal pathways and simultaneous evaluation of multiple parameters for detection of on- and off-target effects of drug interventions. Pathway analysis has commonly been carried out by evaluation of protein expression and/or activation, either by Western blotting for small sample sets or by tissue [25] or gene expression arrays [26] for assessment of multiple signalling nodes. Here, we report on the great potential of phosphoflow cytometry with FCB for the analysis of signal pathways in glioblastoma. This offers a new quantitative approach with improved resolution of dynamic signalling features that so far has been missing from conventional analysis technologies.

We have introduced a protocol that preserves phospho-epitopes in adherent cell lines and primary tumour cells for phosphoflow cytometry and subsequent measurements of multiple properties in mixed populations. The protocol can be applied readily to testing of responsiveness in patient biopsies to various stimuli as shown for EGF-mediated activation of signalling events in our experiments. Signalling data from glioblastoma tumours represented in histograms reveal both high and low responding populations within each sample. Thus, it is clear that the method also has potential for further studies of signal changes in isolated populations or cell subsets. Tracking of subsets may be performed by including additional molecular markers to the analysis array, such as anti-CD133 for identification of tumour stem cells.

In these experiments we have tested a limited array of signalling molecules, however, expanding the panel of antibodies for more extensive investigations is straightforward. We found the method to be reproducible, sensitive and specific in assessing signal pathways in glioblastoma samples and suitable also for drug screening and identification of drug responsiveness as demonstrated using rapamycin. PTEN loss has previously been suggested to mediate sensitivity to rapamycin inhibition, however, in our sample set the highest responses to EGF exposure were found in tumour cells with intact PTEN, indicating up-regulated mTOR activity in these cells. The PTEN-positive tumour cells also responded more strongly to rapamycin than the PTEN-negative U87MG cell line, measured by reduced S6 phosphorylation and reduced proliferation.

Combined with other molecular and genetic screening methods, we propose phosphoflow cytometry as a key molecular tool to establish more effective strategies for patient stratification and development of molecularly effective diagnostics and therapies for glioblastoma. Addressing the heterogeneity of cancer signalling more effectively by identifying signal mediators and subpopulations of tumour cells that may be targeted by specific drugs should be a critical

element in tailoring future patient-specific therapies for glioblastoma multiforme.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Akhavan D, Cloughesy TF, Mischel PS (2010) mTOR signaling in glioblastoma: lessons learned from bench to bedside. *Neuro Oncol* 12:882–889
2. Benito R, Gil-Benso R, Quilis V, Perez M, Gregori-Romero M, Roldan P, Gonzalez-Darder J, Cerda-Nicolas M, Lopez-Gines C (2010) Primary glioblastomas with and without EGFR amplification: relationship to genetic alterations and clinicopathological features. *Neuropathology* 30:392–400
3. Chalhoub N, Baker SJ (2009) PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol* 4:127–150
4. Choe G, Horvath S, Cloughesy TF, Crosby K, Seligson D, Palotie A, Inge L, Smith BL, Sawyers CL, Mischel PS (2003) Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients in vivo. *Cancer Res* 63:2742–2746
5. Cloughesy TF, Yoshimoto K, Nghiemphu P, Brown K, Dang J, Zhu S, Hsueh T, Chen Y, Wang W, Youngkin D, Liau L, Martin N, Becker D, Bergsneider M, Lai A, Green R, Oglesby T, Koletto M, Trent J, Horvath S, Mischel PS, Mellinghoff IK, Sawyers CL (2008) Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma. *PLoS Med* 5:e8
6. Ekstrand AJ, Longo N, Hamid ML, Olson JJ, Liu L, Collins VP, James CD (1994) Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. *Oncogene* 9:2313–2320
7. Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, Hahn WC, Ligon KL, Louis DN, Brennan C, Chin L, DePinho RA, Cavenee WK (2007) Malignant astrocytic glioma: genetics, biology and paths to treatment. *Genes Dev* 21:2683–2710
8. Gallia GL, Rand V, Siu IM, Eberhart CG, James CD, Marie SK, Oba-Shinjo SM, Carlotti CG, Caballero OL, Simpson AJ, Brock MV, Massion PP, Carson BS, Riggins GJ (2006) PIK3CA gene mutations in pediatric and adult glioblastoma multiforme. *Mol Cancer Res* 4:709–714
9. Guertin DA, Sabatini DM (2009) The pharmacology of mTOR inhibition. *Sci Signal* 2:e24
10. Kanu OO, Mehta A, Di C, Lin N, Bortoff K, Bigner DD, Yan H, Adamson DC (2009) Glioblastoma multiforme: a review of therapeutic targets. *Expert Opin Ther Targets* 13:701–718
11. Kreisl TN, Lassman AB, Mischel PS, Rosen N, Scher HI, Teruya-Feldstein J, Shaffer D, Lis E, Abrey LE (2009) A pilot study of everolimus and gefitinib in the treatment of recurrent glioblastoma (GBM). *J Neurooncol* 92:99–105
12. Krutzik PO, Clutter MR, Trejo A, Nolan GP (2011) Fluorescent cell barcoding for multiplex flow cytometry. *Curr Protoc Cytom*. doi:10.1002/0471142956.cy0631s55
13. Krutzik PO, Nolan GP (2006) Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nat Methods* 3:361–368

14. Lurje G, Lenz HJ (2009) EGFR signaling and drug discovery. *Oncology* 77:400–410
15. Masui K, Cloughesy TF, Mischel PS (2012) Review: molecular pathology in adult high-grade gliomas: from molecular diagnostics to target therapies. *Neuropathol Appl Neurobiol* 38:271–291
16. Mellingshoff IK, Wang MY, Vivanco I, Haas-Kogan DA, Zhu S, Dia EQ, Lu KV, Yoshimoto K, Huang JH, Chute DJ, Riggs BL, Horvath S, Liau LM, Cavenee WK, Rao PN, Beroukhi R, Peck TC, Lee JC, Sellers WR, Stokoe D, Prados M, Cloughesy TF, Sawyers CL, Mischel PS (2005) Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med* 353:2012–2024
17. Merlo V, Longo M, Novello S, Scagliotti GV (2011) EGFR pathway in advanced non-small cell lung cancer. *Front Biosci (Schol Ed)* 3:501–517
18. Miller TW, Rexer BN, Garrett JT, Arteaga CL (2011) Mutations in the phosphatidylinositol 3-kinase pathway: role in tumor progression and therapeutic implications in breast cancer. *Breast Cancer Res* 13:224
19. Mischel PS, Shai R, Shi T, Horvath S, Lu KV, Choe G, Seligson D, Kremen TJ, Palotie A, Liau LM, Cloughesy TF, Nelson SF (2003) Identification of molecular subtypes of glioblastoma by gene expression profiling. *Oncogene* 22:2361–2373
20. Ogino S, Galon J, Fuchs CS, Dranoff G (2011) Cancer immunology-analysis of host and tumor factors for personalized medicine. *Nat Rev Clin Oncol* 8:711–719
21. Pende M, Um SH, Mieulet V, Sticker M, Goss VL, Mestan J, Mueller M, Fumagalli S, Kozma SC, Thomas G (2004) S6K1(–/–)/S6K2(–/–) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol* 24:3112–3124
22. Podsypanina K, Lee RT, Politis C, Hennessy I, Crane A, Puc J, Neshat M, Wang H, Yang L, Gibbons J, Frost P, Dreisbach V, Blenis J, Gaciong Z, Fisher P, Sawyers C, Hedrick-Ellenson L, Parsons R (2001) An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten ± mice. *Proc Natl Acad Sci USA* 98:10320–10325
23. Preusser M, de Ribaupierre S, Wohrer A, Erridge SC, Hegi M, Weller M, Stupp R (2011) Current concepts and management of glioblastoma. *Ann Neurol* 70:9–21
24. Shi Y, Gera J, Hu L, Hsu JH, Bookstein R, Li W, Lichtenstein A (2002) Enhanced sensitivity of multiple myeloma cells containing PTEN mutations to CCI-779. *Cancer Res* 62:5027–5034
25. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352:987–996
26. Sugawa N, Ekstrand AJ, James CD, Collins VP (1990) Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *Proc Natl Acad Sci USA* 87:8602–8606