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Abbreviations

53BP1	TP53-Binding Protein 1
A4GALT	Lactosylceramide Alpha 1,4-Galactosyltransferase
AR	Androgen Receptor
ATM	Ataxia Telangiectasia Mutated
ATG9A	Autophagy-Related 9A
ATR	Ataxia Telangiectasia and Rad3 Related
BBB	Blood-Brain Barrier
BCAA	Branched-Chain Amino Acids
BCAT1	Branched Chain Amino Acid Transaminase 1
BRCA1/2	Breast Cancer Type 1/2 Susceptibility Protein
CCNU	Chlorethyl-Cyclohexyl-Nitroso-Urea (Lomustine)
CDK4	Cyclin-Dependent Kinase 4
CDKN2A/B	Cyclin-Dependent Kinase Inhibitor 2A/B
CGC	Cancer Gene Consensus
CHK1	Checkpoint Kinase 1
CHK2	Checkpoint Kinase 2
cMYC	Myelocytoma
CNS	Central Nervous System
CpG	Cysteine-Phosphate-Guanine
CTx	Chemotherapy
DDR	DNA Damage Response
DNA	Deoxyribonucleic Acid
DNA-PKcs	DNA-Dependent Protein Kinase Catalytic Subunit
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular-Signal Regulated Kinase
G6PD	Glucose-6-Phosphate Dehydrogenase
GBM	Glioblastoma Multiforme
GLI	Glioma-Associated Oncogene
GSCs	Glioblastoma Stem Cells
GSEA	Gene Set Enrichment Analysis
GSVA	Gene Set Variance Analysis
Gy	Gray
HER2	Human Epidermal Growth Factor Receptor 2
HIFs	Hypoxia-Inducible Transcription Factors
HR	Homologous Recombination
HSP90	Heat Shock Protein 90
IDH	Isocitrate Dehydrogenase
IFN-γ	Interferon Gamma
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IR	Ionizing Radiation
JAK	Janus Kinase
KPS	Karnofsky Performance Status
LAT1	L-Type Amino Acid Transporter
LEA	Leading Edge Analysis
LIG4	DNA Ligase 4

lncRNAs	Long Non-Coding RNAs
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MDM2	Mouse Double Minute 2 Homolog
MDM4	Mouse Double Minute 4 Homolog
MDSCs	Myeloid-Derived Suppressor Cells
MGMT	O6-Methylguanine-DNA Methyltransferase
miRNAs	microRNAs
MMP9	Matrix Metalloproteinase 9
mRNA	Messenger Ribonucleic Acid
MSigDB	Molecular Signatures Database
mTOR	Mammalian Target of Rapamycin
mTORC1	Mammalian Target of Rapamycin Complex 1
mTORC2	Mammalian Target of Rapamycin Complex 2
NBN	Nibrin
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NHEJ	Non-Homologous End Joining
NO	Nitric Oxide
O6MeG	O^6-Methyl-Guanine
OLIG2	Oligodendrocyte Transcription Factor 2
OS	Overall Survival
OXPHOS	Oxidative Phosphorylation
TP53	Tumorsuppressor Protein TP53
PCA	Principal Component Analysis
PKP	Protein Kinase B
PDGFRA	Platelet-Derived Growth Factor Receptor Alpha
PFS	Progression-Free Survival
pH	Potential of Hydrogen
PIP2	Phosphatidylinositol (4,5)-Bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-Trisphosphate
PI3K	Phosphatidylinositol 3-Kinase
POLA1	DNA Polymerase Alpha 1 Catalytic Subunit
PSMA1	Proteasome 20S Subunit Alpha 1
PSMB3	Proteasome 20S Subunit Beta 3
PSMG1	Proteasome Assembly Chaperone 1
PTEN	Phosphatase and Tensin Homolog
QN	Quinacrine
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RAD51	DNA Repair Protein RAD51 Homolog 1
RB	Retinoblastoma
RCTx	Radiochemotherapy
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
RTx	Radiotherapy
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel
SHH	Sonic Hedgehog
SLC7A11	Solute Carrier Family 7 Member 11

SOX	SRY-Box Transcription Factor
STAT	Signal Transducers and Activators of Transcription
STAT5B	Signal Transducer and Activator of Transcription 5B
TAMs	Tumor-Associated Macrophages
TGF-β	Transforming Growth Factor Betta
TME	Tumor Microenvironment
TMZ	Temozolomide
TTF	Tumor Treating Fields
TXNRD1	Thioredoxin Reductase 1
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
WNT	Wingless-Related Integration Site
γH2AX	Gamma H2A Histone Family Member X

List of publications

Publication I: **Schnöller LE**, Albrecht V, Brix N, Nieto AE, Fleischmann DF, Niyazi M, Hess J, Belka C, Unger K, Lauber K, Orth M. Integrative analysis of therapy resistance and transcriptomic profiling data in glioblastoma cells identifies sensitization vulnerabilities for combined modality radiochemotherapy. Radiat Oncol. 2022 Apr 19;17(1):79. doi: 10.1186/s13014-022-02052-z. PMID: 35440003; PMCID: PMC9020080.

Publication II: **Schnöller LE**, Piehlmaier D, Weber P, Brix N, Fleischmann DF, Nieto AE, Selmansberger M, Heider T, Hess J, Niyazi M, Belka C, Lauber K, Unger K, Orth M. Systematic in vitro analysis of therapy resistance in glioblastoma cell lines by integration of clonogenic survival data with multi-level molecular data. Radiat Oncol. 2023 Mar 11;18(1):51. doi: 10.1186/s13014-023-02241-4. PMID: 36906590; PMCID: PMC10007763.

1. Contribution and declaration

The present work is submitted as a cumulative dissertation and is based on two published original research articles (1, 2), both with Leon Emanuel Käfer (né Schnöller) as first author. The project was primarily supervised, managed and designed by Prof. Dr. Kirsten Lauber and Dr. Michael Orth. All co-authors have given their consent to include these articles in this cumulative dissertation for the degree of Doctor of Medicine (Dr. med.). The co-authors have declared that the submitted articles will not be used in other dissertations by signing the corresponding cumulative dissertation form.

1.1. Contribution to paper I

I, Leon Emanuel Käfer contributed significantly to the investigation, validation and formal analysis of clonogenic survival assays for a panel of seven human glioblastoma (GBM) cell lines following various treatment modalities. To further validate the assessed data, I supported the conceptualization of subsequent experiments and carried out the investigation, validation and formal analysis of clonogenic survival upon pharmacological inhibition of identified regulators of the DNA damage response in combination with irradiation (IR) or temozolomide (TMZ). Finally, I contributed to data curation and visualization and drafted the original manuscript.

1.2. Contribution to paper II

The correlation analyses performed in the second original research article are largely based on the investigation and formal analysis carried out by me, Leon Emanuel Käfer, as presented in the first publication. I contributed to data curation, visualization, and wrote the original draft of the manuscript.

2. Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults, characterized by rapid growth, diffuse infiltration, and marked genetic and phenotypic heterogeneity (3–6). Despite advances in surgery, radiotherapy, and chemotherapy, the prognosis remains dismal, with a median survival of approximately 15 months, underscoring the urgent need for innovative therapeutic strategies (7–9).

2.1. Epidemiology and etiology

Being the most aggressive and prevalent primary brain tumor, GBM accounts for approximately 49.1% of all malignant tumors of the central nervous system (CNS) and 14.3% of all primary brain tumors and other CNS tumors, including benign ones (10–12). Due to its high degree of malignancy, it is classified as grade IV according to the World Health Organization (WHO) grading system (13). In the updated 2021 WHO classification, GBM has been redefined, and the previous differentiation based on isocitrate dehydrogenase (IDH) mutation status has been removed. GBM now exclusively refers to IDH wild-type cases, while astrocytomas with mutated IDH status are classified separately (13).

In Europe and North America, the incidence of GBM is approximately 3.05 to 3.55 new cases per 100,000 people (12, 14). Although the majority of cases occur sporadically without a clearly identifiable cause, GBM is associated with several known risk factors (15–17). Age and sex are significant factors, since GBM is more common in older adults, with incidence peaking between 65 and 75 years (12, 14, 18), and it is slightly more frequent in males than females (4.04 new cases compared to 2.53 new cases per 100,000 people) (12). Certain hereditary conditions, such as Li-Fraumeni syndrome, Lynch syndrome, Turcot syndrome, and neurofibromatosis type 1, are linked to an increased risk of GBM due to mutations in tumor suppressor genes or deoxyribonucleic acid (DNA) repair pathways (15, 19, 20). Exposure to high-dose ionizing radiation, whether from therapeutic treatments to the head or accidental exposure, is a well-established risk factor for gliomas, including GBM (15–17). While the role of lifestyle and environmental factors is less clearly defined, some studies suggest a potential link to long-term exposure to pesticides or petrochemicals (21–23). Additionally, GBM can develop as a progression from lower-grade gliomas, a process often associated with specific genetic changes, such as TP53 mutations and IDH wild-type status (24, 25). Ethnicity also appears to play a role, since GBM is more frequently diagnosed in individuals of Caucasian descent compared to other ethnic groups (26, 27). Despite these factors, the majority of GBM cases arise without a clear underlying cause, underscoring the complexity of its development.

2.2. Current standard of care

GBM is a highly aggressive and lethal cancer (28, 29). 5-year survival remains limited to 5-10% with a median overall survival (OS) of 15 months (12, 30, 31). Despite intensive research, this dismal prognosis failed to improve over the past decades (32). Only few prognostic factors are available, including age, Karnofsky performance status (KPS), tumor localization, and extent of surgery/biopsy. These can be used to classify patients into four risk groups. Tumor localization is most relevant in younger patients when assessing low or low-to-moderate risk. Briefly, low-risk patients (age ≤ 40 years, frontally located tumor) exhibit a 2-year survival rate of 65%, whereas the high-risk group (age ≥ 65 years or poor KPS or tumor biopsy only) is associated

with a considerably lower 2-year survival rate of 2% (33). In conjunction with the molecular pathology profile, these prognostic factors should be considered when making treatment decisions (34). Standard treatment protocols involve maximal safe resection of the tumor mass followed by six weeks of concurrent treatment with temozolomide (TMZ) and radiotherapy (RTx) with a cumulative dose of 60 Gray (Gy). Subsequently, adjuvant TMZ treatment is continued for six cycles (30, 31).

As an alkylating agent, TMZ induces cross-linking and strand breaks of nucleic acids (deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)) via the formation of O^6-methyl-guanine (O6MeG) lesions, thereby disrupting DNA synthesis and potentially triggering cell death mechanisms (35, 36). Patients with evidence of cysteine-phosphate-guanine (CpG) island hypermethylation of the promoter region of the DNA damage repair (DRR) enzyme O6-methylguanine-DNA methyltransferase (MGMT) exhibit improved response to TMZ (37–39). Conversely, the cytotoxic effect of TMZ is impaired in the absence of MGMT promoter methylation, emphasizing the role of MGMT methylation status as an essential predictive marker (40, 41). Although methylation of the MGMT promoter appears homogeneous within a tumor, changes in methylation status may occur at recurrence, warranting reassessment when appropriate (42).

In addition to standard of care, treatment with tumor treating fields (TTF) presents a promising therapeutic option after completion of radiochemotherapy (RCTx), potentially leading to prolonged progression-free survival (PFS) (hazard ratio 0.63; median 2.7 months) and OS (hazard ratio 0.63; median 4.9 months) compared to TMZ alone (8, 43). In younger patients (≤ 70 years) with excellent performance status and evidence of MGMT promoter methylation, a combination of the nitrosourea CCNU (lomustine) in addition to RCTx with TMZ may result in prolonged median OS (hazard ratio 0.6, 31.4 months vs. 48.1 months) (44). However, pseudoprogression and hematologic toxicity are potential drawbacks (45). In relapse, especially after failure of first-line therapy, CCNU is commonly utilized (46, 47), although drug-induced thrombocytopenia remains a major limitation (48). The addition of the anti-vascular endothelial growth factor (VEGF) monoclonal antibody, bevacizumab (Avastin), to RCTx may prolong PFS (10.7 months vs. 7.3 months; hazard ratio for progression or death, 0.79) and steroid-free time, but not OS (49). In relapsed stages and re-irradiation scenarios, bevacizumab may prevent/improve radiation necrosis or edema-related symptoms (50, 51), but again without impacting OS (52).

Despite advances in cancer immunotherapy, immune-based therapies have not proven effective in newly diagnosed and relapsed GBM (53, 54). This could be due to the immunologically privileged location, the immunosuppressive environment of GBM (55, 56), the reduced frequency of somatic mutations, and resistance mechanisms to checkpoint inhibitors (57).

The lack of standardized recommendations for recurrent GBM further complicates treatment decisions and requires individualized assessment to determine the most appropriate course of action. Possible options may include resection, re-irradiation, or chemotherapy/targeted therapy, respectively (46, 58).

2.3. Mechanisms of treatment resistance

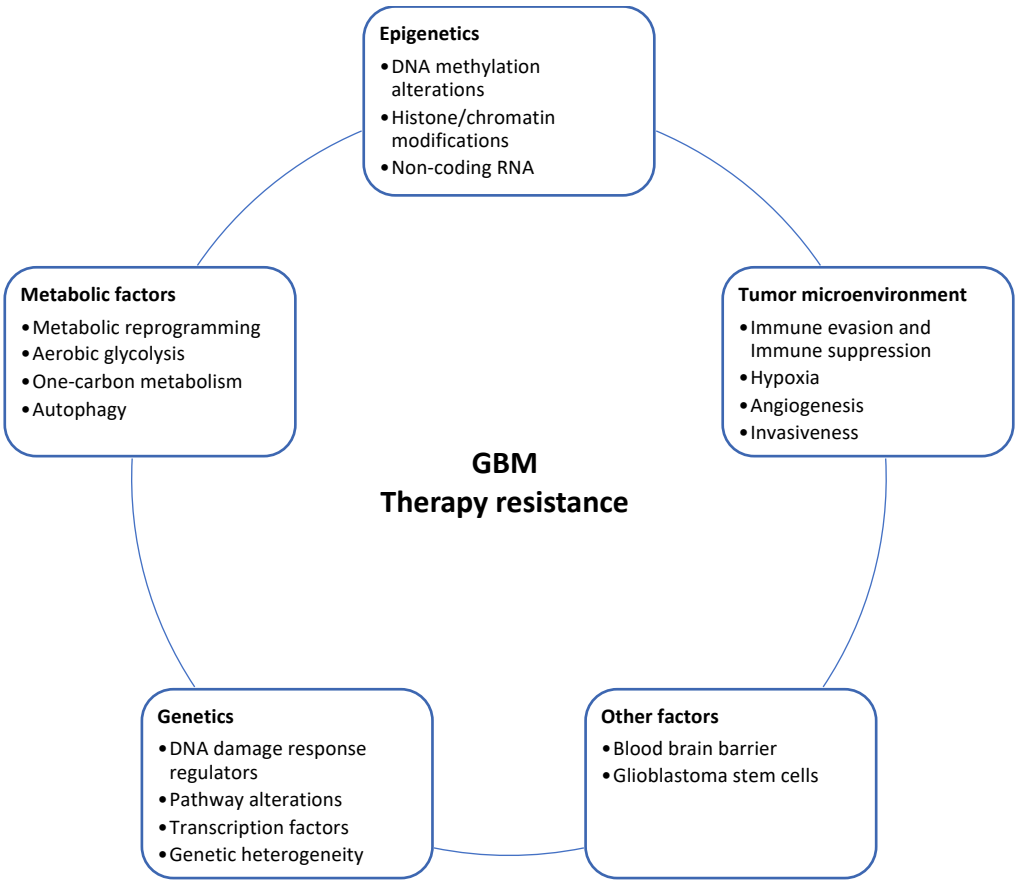

Figure 1: Mechanisms of treatment resistance in GBM.

GBM exhibits strong inherent treatment resistance, leading to limited response and frequent treatment failure with an average recurrence at only seven months after multimodal standard-of-care treatment (7). The infiltrating growth pattern often precludes complete resection despite high-quality intraoperative imaging and fluorescein guidance without risking severe cognitive impairment by compromising healthy brain parenchyma (59–61). Due to extensive inherent resistance, the response to RCTx is significantly impaired, resulting in local recurrence within the previously treated area in approximately 80-90% of cases (62–64) (Figure 1). Moreover, less than half of the patients exhibit an adequate response to TMZ (65, 66), and the onset of adaptive resistance, occurring in over 90% of cases, further attenuates the drug's efficacy upon reapplication in cases of recurrent GBM (67). The unique tumor microenvironment and the presence of the blood-brain barrier additionally limit the accessibility of many therapeutics, resulting in the failure of several experimental drugs subjected to clinical trials (68) (Figure 1).

2.3.1. Insights into the molecular complexity underpinning treatment resistance

To understand the complex molecular landscape of GBM and to advance the treatment of GBM, the genomics and transcriptomics of GBM have been extensively studied, with whole genome and transcriptome sequencing of both primary and recurrent GBM documenting the coexistence of distinct molecular subtypes and delineating inter- and intratumoral heterogeneity hallmarks of treatment resistance (69) (Figure 1). A comprehensive molecular

characterization of 206 GBM tumors revealed that three major pathways are altered, the receptor tyrosine kinase (RTK)/RAS/phosphatidylinositol 3-kinase (PI3K) pathway in 88% of patients, the tumorsuppressor protein TP53 pathway in 87% of patients, and the retinoblastoma (RB) pathway in 78% of patients, with frequent genetic amplification of epidermal growth factor receptor (EGFR), cyclin-dependent kinase 4 (CKD4), platelet-derived growth factor receptor alpha (PDGFRA), mouse double minute 2 homolog (MDM2), and mouse double minute 4 homolog (MDM4). The most prevalent genetic deletions were found in cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B) and phosphatase and tensin homolog (PTEN) (70). These alterations in GBM perturb the regulatory mechanisms of cell growth, repair, and cell death, enhancing the tumor's resistance to treatment. Central to this is the RTK/RAS/PI3K pathway, which is involved in the regulaton of cell growth, survival, and proliferation. When dysregulated, it can promote uncontrolled cell growth and inhibit cell death, rendering therapeutic approaches ineffective (71). In addition, aberrant activation of mammalian target of rapamycin (mTOR), a downstream effector in this pathway, further enforces uncontrolled cell growth. mTOR acts through two complexes: mammalian target of rapamycin complex 1 (mTORC1), which is activated by AKT to drive protein synthesis and growth, and mammalian target of rapamycin complex 1 (mTORC2), which enhances AKT activity, forming a feedback loop in the PI3K pathway (71–73). In turn, mTOR inhibitors have been developed to target tumors with hyperactive PI3K/AKT/mTOR signaling, such as GBM (74). The TP53 pathway is critical for DNA repair, cell cycle regulation and apoptosis. Deregulations here can affect DNA repair, cancer cell stemness and evasion of cell death, impairing the response to DNA-damaging treatments, such as IR or TMZ (75, 76). Likewise, malfunction of the RB pathway can accelerate cell division, supporting tumor growth and reducing the efficacy of treatments (77). Amplification of EGFR, CKD4, PDGFRA, and MDM2 can fuel unrestrained cell proliferation, growth and survival, resulting in more aggressive tumors that become less responsive to treatment (78–81). Key to cell cycle regulation is CDKN2A/B, while PTEN acts as counterregulator to the PI3K/AKT/mTOR pathway by dephosphorylating PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) to PIP2 (phosphatidylinositol (4,5)-bisphosphate), which in turn suppresses AKT and mTOR activation (82). Loss of these regulators can enhance cell growth and therapy resistance (83, 84).

Besides the discussed mechanisms, several other pathways and transcription factors promote GBM treatment resistance by maintaining stem cell properties or by upregulating the DDR, repectively (85) (Figure 1). In this context, extracellular signaling pathways, such as WNT/β-catenin, transforming growth factor-β (TGF-β)-SOX4-SOX2, HEDGEHOG and NOTCH, are key contributors. For example, the WNT/β-catenin signaling pathway promotes cell proliferation, invasion, angiogenesis, and stemness (86–89), while the TGF-β signaling pathway drives the expression of SOX4 and SOX2, which maintain stem cell-like properties and promote tumor progression (90–92). Similarly, the HEDGEHOG and NOTCH pathways, known for their roles in tumor growth, maintenance of glioblastoma stem cells (GSCs) and therapy resistance, are frequently dysregulated in GBM (93, 94).

Another important contributor is the SONIC HEDGEHOG (SHH)/ glioma-associated oncogene (GLI) signaling axis, which supports tumor stemness and therapy resistance through activation of the GLI family of transcription factors (95, 96). In addition, the JAK/STAT pathway, which also maintains GBM stem cell properties and enhances immune evasion, further strengthens GBM survival mechanisms under therapeutic stress (97–101).

In promoting therapy resistance, transcription factors also play a pivotal role (Figure 1). Specifically, the stem cell transcription factors oligodendrocyte transcription factor 2 (OLIG2) and c-MYC (abbreviation for myelocytoma) drive self-renewal and proliferation of GSCs,

thereby contributing to tumor growth and survival (102–104). NANOG, a regulator of stem cell pluripotency and regulated by SHH-GLI, promotes self-renewal and stem-like behavior (105), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediates many hallmarks of cancer, including inflammatory and stress responses that support tumor survival (106, 107). Similarly, SOX and OCT4 enhance the stemness and self-renewal capacity of GSCs, making them central to tumor aggressiveness and resistance to conventional therapies (92, 108).

In addition to the discussed signaling pathways and transcription factors, epigenetic modifications further complicate therapeutic strategies by altering the transcriptional landscape of the tumor, thereby orchestrating key processes such as tumor survival, DDR, autophagy stimulation, and stemness maintenance (Figure 1). Alterations in DNA methylation patterns can silence tumor suppressor genes or activate proto-oncogenes (109), while non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) have been shown to regulate various biological processes in GBM, including tumor initiation, progression and survival (110, 111). Histone modifications and chromatin remodeling may additionally affect gene accessibility and transcriptional activity, thereby contributing to adaptive therapeutic resistance (109).

2.3.2. Failure of DNA-damaging treatment in the light of upregulated DNA damage repair mechanisms in GBM

The DNA damage response (DDR) is another major driver of treatment resistance in GBM (Figure 1). While the DDR is essential for maintaining genomic integrity and preventing cancer progression, its upregulation in GBM cells enhances resistance to DNA-damaging treatments and represents a therapeutic obstacle (1, 112–114). Key regulators, such as ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3 related (ATR) kinases, orchestrate the DDR. ATM responds to DNA double-strand breaks, while ATR is activated by single-strand breaks and stalled replication forks, both of which trigger a cascade of cellular responses, including cell cycle arrest, DNA repair, and cell death. The checkpoint kinases CHK1 and CHK2 function downstream of ATR and ATM, respectively, facilitating cell cycle arrest and DNA repair (115, 116). Non-homologous end joining (NHEJ) and homologous recombination (HR) are pivotal pathways for the repair of severe DNA double-strand breaks and overexpression of key proteins in these pathways, such as DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and DNA ligase 4 (LIG4) for NHEJ and RAD51 and breast cancer type 1/2 susceptibility protein (BRCA1/2) for HR, enhances GBM resistance to DNA-targeted treatments (1, 117–121).

2.3.3. Beyond genetics: The intricate interplay of the tumor microenvironment (TME) and glioblastoma stem cells (GSCs)

Focusing primarily on tumor cells may leave the crucial role of the tumor microenvironment (TME) in influencing treatment resistance unrecognized, possibly limiting therapeutic progress (122–124) (Figure 1). The GBM TME is defined by distinct features, including endothelial cells and tumor-associated macrophages (TAMs) in the perivascular regions, as well as hypoxic and infiltrative zones, collectively referred to as tumor niches (122). While GSCs were initially thought to reside specifically within these niches, recent research indicates a broader distribution in the tumor tissue and an intricate crosstalk with different cell types of the TME (125). Indeed, GSCs play a pivotal role in shaping the TME by secreting factors that attract and

remodel their cellular components and stimulate angiogenesis, while the TME promotes GSCs maintenance and development. This dynamic interplay continually refines the tumor ecosystem and is critical for GBM progression, treatment resistance, and recurrence (122, 124, 126, 127).

2.3.4. Fueling inflammation, favoring immune evasion: The role of the perivascular tumor microenvironment (TME) in treatment resistance

The perivascular niche is characterized by a dynamic network of astrocytes, fibroblasts, immune cells, glioma stem cells, and pericytes (128). GBM cells stimulate abnormal angiogenesis via VEGF, forming disorganized and leaky blood vessels that compromise the integrity of the blood-brain barrier (BBB) (129, 130). Attracted by the local inflammation induced by GBM cells, immune cells, including neutrophil granulocytes, monocytes, myeloid-derived suppressor cells (MDSCs), and TAMs, infiltrate the perivascular niche through the permeable BBB, thereby enhancing angiogenesis and establishing an immunosuppressive environment. Moreover, these immune cells interact with GSCs and tumor cells, supporting tumor growth and treatment resistance (131–133). TAMs, the predominant infiltrating immune cells, exhibit either the tumor-suppressing type-1 phenotype of classically activated macrophages (M1) induced by lipopolysaccharides (LPS) and IFN-γ, or the glioma-promoting type-2 phenotype of alternatively activated macrophages (M2) induced by interleukin-4 (IL-4) and IL-13 (134, 135). TAMs, typically situated near GSCs, are recruited by various chemoattractants and release TGF-β, which elevates matrix metalloproteinase 9 (MMP9) expression and enhances GSCs invasiveness (136). The extracellular matrix protein periostin, released by GSCs, acts as a chemoattractant and correlates with tumor grade and recurrence (137, 138).

2.3.5. Hypoxic signaling and metabolic reprogramming mediate treatment resistance

Conversely, the tumor niche is located in hypoxic areas with inconsistent oxygen supply due to inadequate vascularization and frequently occluded blood vessels (122). While necrosis occurs within these insufficiently supplied areas, marginal hypoxic glioma cells form pseudopalisades - a hallmark of GBM - and actively migrate away from the necrotic region (139, 140). Although poor vascularisation, hypoxia, and necrosis may seem counterintuitive to tumor growth, they are associated with poor prognosis and mark the characteristic transition from high-grade astrocytoma to GBM (141). Indeed, hypoxia intensifies chemotherapy resistance not only by inducing an acidic pH that inactivates pH-sensitive drugs but also by isolating the tumor niche cells from blood vessels, significantly impeding anticancer drugs from reaching hypoxic areas and thus, markedly reducing their efficacy (142–144). Moreover, in vitro studies reveal that under hypoxic conditions, GBM cells demonstrate elevated cell migration, upregulation of hypoxia-related genes, and elevated treatment resistance (145). Tumor cells in this niche are highly adapted to hypoxia and are resistant to cell death, while the tumor environment has been documented to influence tumor survival and progression in multiple ways (146). These include the metabolic shift to aerobic glycolysis, commonly known as the Warburg effect, and one-carbon metabolism, as well as increased amino acid metabolism and fatty acid oxidation to meet high metabolic demands (147–149). However, hypoxia also facilitates the maintenance of stem cell properties, promotes tumor growth, and creates a protective ecosystem that provides limited immune surveillance and sustained cell survival despite of radio-, immuno-, or chemotherapy (150–154). Since IR predominantly causes indirect DNA

damage and genetic instability through the generation of reactive oxygen species (ROS) (155, 156), cells of the hypoxic tumor niche exhibit strong inherent radioresistance mediated predominately by autophagy and hypoxia-inducible transcription factors (HIFs) (157) (Figure 1). HIFs, stabilized by PI3K-AKT activation and hypoxia, act as pivotal transcription factors for numerous gene products (158, 159). Their expression is further amplified by the frequently abnormal activation of mitogen-activated protein kinase (MAPK)/ extracellular-signal regulated kinase ERK or JAK2/ STAT3 signaling pathways in GBM cells (160). Specifically, HIF-1α orchestrates metabolic processes by augmenting glucose uptake and amino acid metabolism while modulating various cellular processes, including cell proliferation and mobility (161). It enhances angiogenesis through amplifying VEGF expression (162) and influences oncogenic transcriptional pathways, including NOTCH signaling, EGFR, and MAPK/ERK, which directly impact GSCs maintenance, tumor cell migration, invasiveness, and survival (162–165). HIF-1α also markedly expresses inducible nitric oxide synthase (iNOS), a key enzyme that transforms L-arginine into nitric oxide (NO), thereby modulating radioresistance and governing various cellular processes, such as regulation of cell cycle, angiogenesis, and tumor cell survival (166–169). HIF-1α and HIF-2α increase the expression of L-type amino acid transporter 1 (LAT1, also known as SLC7A5), a transporter for branched-chain amino acids (BCAA), and branched chain amino acid transaminase 1 (BCAT1), a metabolic enzyme for BCAA, thus enhancing amino acid metabolism (170). Additionally, HIF-1α enhances the expression of DDR regulators, including MGMT, strengthening radio- and chemoresistance (171–174). HIF-1α expression in TMZ-resistant GBM cells is likely steered by the human epidermal growth factor receptor 2 (HER2)-dependent PI3K/AKT/mTOR pathway (175). Moreover, the reciprocal activation of CDKN1A and HIF-1α enforces radioresistance through a positive feedback loop (176). Autophagy, a conserved catabolic process downstream of mTOR hyperactivation, replenishes components of damaged or unusable proteins into the nutrient cycle, strengthening tumor cell survival amidst resource deprivation and sustained oxidative stress (177–179). In GBM cells, this mechanism is enhanced by hypoxic conditions and aberrant activation of NOTCH, WNT/β-catenin, and HEDGEHOG signaling pathways, together with autophagy-related 9A (ATG9A), fueling therapy resistance and sustaining GSCs by furnishing an energy source (180–182). At the same time, inhibition of autophagy with quinacrine (which increases lysosomal pH) and TMZ (which is known to stimulate autophagy) has been demonstrated to render GSCs susceptible to TMZ via accumulation of autophagic vacuoles (183). Besides enhancing the cytotoxicity of TMZ, it may also cause mitochondrial damage through peroxidation of membrane lipids, a hallmark of ferroptosis (183) - a form of nonapoptotic cell death characterized by Fe(II)-dependent lipid peroxidation without oxidative stress-driven ROS accumulation (184, 185).

2.3.6. Tumor dynamics and temporal and spatial heterogeneity as a hallmark of treatment resistance

In the vascular invasive tumor niche, infiltration occurs either of individual tumor cells or collectively along white matter or basement membrane pathways - as along vessels - into the adjacent healthy brain parenchyma (122, 186, 187). Noteworthy, invasion within the vascular invasive tumor niche happens independently of MMPs (188). Invasiveness contributes significantly to the strong temporal and spatial heterogeneity and describes a hallmark of treatment resistance (6, 69); cells that infiltrate healthy brain parenchyma evade surgery or radiation and induce a new cycle of necrotic and vascular proliferative areas (122) (Figure 1).

2.4. Targeting GBM's treatment resistance

GBM exhibits robust inherent treatment resistance mechanisms that frequently lead to therapeutic failure, facilitate relapse, and hamper the advancement of novel therapeutic strategies. Consequently, amidst ongoing efforts to refine GBM treatment, the mechanisms underlying treatment resistance have moved into the focus of GBM research (167–169). Despite their promise, small molecule inhibitors have achieved limited clinical success in the treatment of GBM. Challenges include non-specific targeting resulting in significant toxicity and side effects, the BBB preventing optimal drug concentrations within the CNS, and the inherent immune-privileged nature of the CNS hindering immune-based therapies (Figure 1). While many GBM inhibitors focus on kinases, only a few, such as specific EGFR (AZD3759 and NT113) and PI3K (GDC-0084) inhibitors, have been designed for effective brain penetration (92). To be effective, a CNS drug must not only have strong target specificity but also demonstrate optimal brain permeability. The drug's efficacy is further influenced by the complex interplay between its therapeutic targets, the TME, and the inherent features of cancer cells. These cancer cells exemplify the marked intra- and intertumoral heterogeneity seen in GBM, underscoring that a one-size-fits-all therapeutic approach may not be sufficient (170). Combined modality therapy is emerging as a potential solution that aims to address this heterogeneity by simultaneously targeting both pre-existing tumor cell populations and evolving resistant clones. Such an approach may minimize the chances of treatment failure and improve therapeutic outcomes. Indeed, previous findings from our research group have demonstrated that inhibiting heat shock protein 90 (HSP90), crucial for the proper folding and functionality of numerous gene products, including DDR regulators, induces potent radiosensitization in soft tissue sarcomas (171), colorectal tumors (172), and most recently in GBM (90).

2.5. Objectives and key findings of this thesis

The pronounced resistance of glioblastoma (GBM) to conventional therapies, driven by complex molecular mechanisms and an immunosuppressive microenvironment, underscores the urgent need for innovative therapeutic strategies. To this end, the present work aimed to advance the understanding of GBM treatment resistance while expanding the range of actionable resistance regulators for small-molecule inhibitor-driven radio-/chemosensitization and their potential use as predictive biomarkers.

2.5.1. Paper I: Integrative analysis of therapy resistance and transcriptomic profiling data in glioblastoma cells identifies sensitization vulnerabilities for combined modality radiochemotherapy

To identify new strategies for optimized TMZ-based radiochemotherapy of GBM, we designed an integrative approach combining clonogenic survival-based resistance scores with quantitative real-time PCR (qRT-PCR)-derived transcriptomic expression profiles of DDR regulators from a well-established panel of human GBM cell lines. This approach identified numerous DDR targets, including PARP1 and HSP90, both supported by readily available advanced pharmacological inhibitors and their well-documented capacity to facilitate radio- and chemosensitization (112, 189–193). Additionally, novel targets emerged, including ATR and LIG4 for radiosensitization and ATM for sensitization to TMZ treatment. Although no radiosensitizing effect was observed for the poly-DNA ligase inhibitor L189, AZD-6738-

mediated pharmacological inhibition of ATR resulted in potent and significant radiosensitization. ATM inhibition with KU-60019 led to a cell line-dependent sensitization to TMZ treatment which did not reach statistical significance. Collectively, these results validate the feasibility of our integrative approach and highlight its potential to unravel actionable therapeutic targets, while at the same time revealing the limitations of its correlative nature.

2.5.2. Paper II: Systematic in vitro analysis of therapy resistance in glioblastoma cell lines by integration of clonogenic survival data with multi-level molecular data

Subsequently, we used the previously assessed resistance scores in a multi-level integration workflow with global microarray-based transcriptional data of the same GBM cell line panel to identify potential drivers of treatment resistance in various biological processes. This included correlation analyses at the single gene level as well as a targeted analysis of genes preselected from the Cancer Gene Consensus (CGC) collection (194). Indeed, susceptibility to TMZ treatment showed the strongest correlation with MGMT expression, confirming our hypothesis that drivers of treatment resistance can be systematically identified using this integrative approach. Furthermore, our approach identified numerous druggable targets, such as the androgen receptor (AR), mitogen-activated protein kinase 4 (MAP2K4), and signal transducer and activator of transcription 5B (STAT5B). Gene set enrichment analysis (GSEA) and gene set variance analysis (GSVA) using the molecular signatures database (MSigDB) Hallmarks collection further linked overarching biological processes to treatment resistance (195). Out of the 50 hallmark gene sets, ROS pathway was the only one to correlate with radiation resistance in both modes examined (single-shot and fractionated IR), while MTORC1 signaling showed the most positive and TNF-alpha signaling via NF-kB the most negative correlating intersections with treatment resistance. Leading edge analyses (LEA) were performed to identify key contributors within these three enriched gene sets, and ROS detoxification emerged as the most promising target for sensitization in combined treatment approaches, given the availability of pharmacological agents. Druggable targets identified include genes involved in thioredoxin/peroxiredoxin metabolism (e.g., TXNRD1, PRDX1) and glutathione (GSH) synthesis (e.g., GSR, ABCC1, GCLC).
In addition to these findings, the study provides a comprehensive, multi-omics characterization of the used GBM cell line panel, encompassing transcriptomics, epigenomics, and genomics. Via multi-level cytogenetic and molecular characterization, the cell lines have been classified into classical, proneural, and mesenchymal subtypes, with some exhibiting mixed and/or transitional features most likely due to chromosomal instability (CIN) and the molecular and phenotypic plasticity of GBM. However, our data revealed no clear association between treatment resistance, major transcriptomic differences, and/or a distinct molecular subtype, underscoring the complexity of GBM biology.

2.5.3. Closing knowledge gaps: contributions to glioblastoma research

Given the limited efficacy of current treatment options for glioblastoma (GBM), the development of innovative therapeutic strategies is critical to improving patient survival. Understanding the mechanisms underlying treatment resistance is essential for the development of targeted combined modality therapies to address these challenges (196–198). The present work validates previous findings on a variety of known drivers of treatment resistance, including MGMT (40), PARP1, and HSP90, where pharmacological inhibition has demonstrated radio-/chemosensitization (112, 189–193). In addition, we achieved significant

radiosensitization with the BBB-penetrant agent AZD6738 targeting ATR, a DDR regulator associated with radioresistance and still underrepresented in research (199, 200). Another promising therapeutic target identified is the androgen receptor (AR), which has been successfully targeted in prostate cancer and warrants further investigation in GBM (201–203). Our results provide proof-of-concept for the integrative approach used in this work, which not only validates established targets but also identifies novel ones, providing a framework that can be applied to other tumor entities and large molecular datasets for therapeutic advances.

3. Summary

Background: Despite immense clinical and preclinical efforts, the prognosis of GBM has not been improved over decades, emphasizing the need to discover and establish new therapeutic options. A major obstacle in this regard is the strong inherent therapy resistance related to enhanced DDR signaling, subclonal heterogeneity, adaptation to hypoxia, and ferroptotic and autophagic regulatory hubs. To this end, the present thesis was conceptualized to comprehensively analyze treatment resistance of GBM at multiple molecular levels in vitro and to develop an integrative approach by which potential vulnerabilities for targeted optimization of radio- /chemotherapy can be identified.

Methods: The first publication assessed the inherent treatment resistance of selected well-established human GBM cell lines by measuring clonogenic survival upon treatment with single or fractionated IR and TMZ treatment w/optional concurrent IR. Clonogenic survival data were subjected to principal component analysis (PCA) using the first principal component as the respective resistance score to the corresponding treatment. The mRNA expression levels of 38 DNA damage response regulators were assessed by quantitative real-time polymerase chain reaction (qRT-PCR) and subsequently subjected to correlation analysis with the obtained resistance scores. Determination of MGMT expression was based on sodium dodecyl sulfate – polyacrylamide gel (SDS-PAGE) and Western blot analyses, whereas methylome arrays assessed the methylation status of the MGMT promoter. The top hit candidates were further validated by determining clonogenic survival and DNA repair kinetics in response to (chemo-)irradiation by staining gamma H2A histone family member X (γH2AX)/ TP53-Binding Protein 1 (53BP1) foci following pharmacological inhibition. For the second publication, cell line characterization was enriched with multi-level molecular data, including spectral karyotyping, array-based comparative genomic hybridization, DNA methylation analysis, and microarray-based global gene expression profiling. To break down treatment resistance from single gene to gene set/pathway level, a multistep integration of our clonogenic survival-based resistance scores with transcriptomic profiling data was performed, followed by gene set enrichment analysis (GSEA), gene set variance analysis (GSVA), and leading edge analysis (LEA).

Results: Examined cell lines exhibited variable but substantial treatment resistance, with those deficient in MGMT expression responding to TMZ, as expected. Consistently, there was robust upregulation of DDR regulators and corresponding correlation with treatment resistance, including PARP1, HSP90AB1, and nibrin (NBN), for which pharmacological inhibitors are readily available and whose impact in GBM is well-accepted. ATR and LIG4 emerged as previously underappreciated DDR regulators related to radioresistance, while ATM expression correlated with chemoresistance. Functional validation showed strong radiosensitization upon inhibition of ATR, in contrast to LIG4, and documented slight but non-significant sensitization to TMZ treatment upon inhibition of ATM. In the second publication, the spectrum of potential targets for pharmacological inhibition was comprehensively broadened. Integrating resistance scores with global transcriptome profiling data pinpointed 14 molecular correlates with treatment resistance, such as proteasome 20S subunit beta 3 (PSMB3), lactosylceramide alpha 1,4-galactosyltransferase (A4GALT), and DNA polymerase alpha 1 catalytic subunit (POLA1). By matching our mRNA expression data with the Cancer Gene Consensus (CGC) gene collection, we identified androgen receptor (AR), STAT5B, and MAP2K4, which positively correlate with treatment resistance and are known contributors to GBM progression, with readily available inhibitors. At gene set level, GSEA identified 21 positively and 14 negatively enriched gene sets,

with the ROS pathway, mTORC1 signaling, and TNFα via nuclear factor kappa B (NF-κB) signaling emerging as key drivers of inherent treatment resistance, implicated in the ROS-autophagy-ferroptosis axis. This confirmed the involvement of the ROS-autophagy-ferroptosis axis in treatment resistance, and LEA of these gene sets revealed 14 potential drug targets, including glucose-6-phosphate dehydrogenase (G6PD), solute carrier family 7 member 11 (SLC7A11), proteasome assembly chaperone 1 (PSMG1), proteasome 20S subunit alpha 1 (PSMA1), and thioredoxin reductase 1 (TXNRD1).

Conclusion: In this study, we provide an in-depth investigation of the molecular drivers behind therapy resistance in human GBM cells, broadening the scope for targeted therapeutic strategies and validating the efficacy and feasibility of our multi-level integrative approach. The data sets supporting this work are of potential value for in-depth research in treatment resistance. Beyond this, our systematic workflow can be readily applied to other data sets and tumor types.

4. Zusammenfassung

Hintergrund: Trotz intensiver klinischer und präklinischer Bemühungen hat sich die Prognose des Glioblastoms über Jahrzehnte nicht verbessert, was die Dringlichkeit unterstreicht, neue Therapieoptionen zu identifizieren und zu evaluieren. In diesem Kontext stellt die starke inhärente Therapieresistenz ein signifikantes Hindernis dar, das mit verstärkter DNA-Schadensantwort, subklonaler Heterogenität, der Anpassung an Hypoxie sowie ferroptotischen und autophagischen regulatorischen Achsen in Zusammenhang steht. Die vorliegende Arbeit wurde konzipiert, um die Behandlungsresistenz von Glioblastomzellen auf mehreren molekularen Ebenen in vitro umfassend zu analysieren und einen integrativen Ansatz zu entwickeln, mit dem potenzielle Schwachstellen für eine gezielte Optimierung der Radiochemotherapie identifiziert werden können.

Methoden: In der ersten Publikation wurde die inhärente Behandlungsresistenz ausgewählter etablierter humaner Glioblastomzelllinien durch Messung des klonogenen Überlebens nach Behandlung mit einzeitiger oder fraktionierter Bestrahlung sowie nach Temozolomid-Behandlung mit optionaler gleichzeitiger Bestrahlung evaluiert. Die Daten zum klonogenen Überleben wurden einer Hauptkomponentenanalyse unterzogen, wobei die erste Hauptkomponente als Resistenzwert für die entsprechende Behandlung verwendet wurde. Die mRNA-Expressionsniveaus von 38 Regulatoren der DNA-Schadensantwort wurden durch quantitative qRT-PCR bewertet und anschließend einer Korrelationsanalyse mit den erhaltenen Therapie-Resistenzwerten unterzogen. Die Bestimmung der MGMT-Expression basierte auf SDS-PAGE und Western-Blot-Analysen, während Methylom-Arrays den Methylierungsstatus des MGMT-Promotors bewerteten. Die Top-Treffer wurden weitergehend validiert, indem das klonogene Überleben und die DNA-Reparaturkinetik nach Radio(chemo)therapie durch Anfärben von γH2AX/TP53-BP1-Foci bestimmt wurden. Für die zweite Publikation wurde die Zellliniencharakterisierung um multi-level molekulare Daten erweitert, darunter spektrale Karyotypisierung, Array-basierte vergleichende genomische Hybridisierung, DNA-Methylierungsanalyse und Microarray-basierte Genexpressionsprofile. Um die Behandlungsresistenz vom einzelnen Gen bis zur Ebene der Gensets/Signalwege aufzuschlüsseln, wurde eine multi-level Integration unserer auf dem klonogenen Überleben basierenden *Resistance Scores* mit transkriptomischen Profildaten durchgeführt, gefolgt von einer *Geneset-Enrichment*-Analyse(GSEA), einer *Geneset-Variation*-Analyse (GSVA) und einer *Leading-Edge*-Analyse (LEA).

Ergebnisse: Die untersuchten Zelllinien wiesen eine variable, jedoch signifikante Behandlungsresistenz auf, wobei diejenigen mit niedriger MGMT-Expression, wie erwartet, auf TMZ ansprachen. Zudem konnte eine konsistente, robuste Hochregulierung von DNA-Schadensreparatur-Regulatoren beobachtet werden, die in einer entsprechenden Relation zur Behandlungsresistenz stand. Hierzu zählten u.a. PARP1, HSP90AB1 und NBN, für die pharmakologische Inhibitoren bereits verfügbar sind und deren Bedeutung beim Glioblastom allgemein anerkannt ist. Darüber hinaus konnten ATR und LIG4 im Zusammenhang mit der Strahlenresistenz bzw. ATM-Expression mit der Chemoresistenz als bisher unterschätzte DNA-Schadensreparatur-Regulatoren identifiziert werden. Die funktionelle Validierung ergab eine starke Radiosensibilisierung nach der Hemmung von ATR (anders als bei LIG4) und dokumentierte eine leichte, allerdings nicht signifikante Sensibilisierung für TMZ-Behandlung nach ATM-Hemmung. In der zweiten Publikation wurde das Portfolio potenzieller Ziele für die pharmakologische Hemmung umfassend erweitert. Durch die Integration von *Resistance*

Scores mit globalen Transkriptom-Daten wurden 14 molekulare Korrelatoren der Behandlungsresistenz ermittelt, darunter PSMB3, A4GALT und POLA1. Der Abgleich unserer mRNA-Expressionsdaten mit der CGC Gensammlung ermöglichte zudem die Identifizierung von AR, STAT5B und MAP2K4, die mit der Therapieresistenz korrelieren und bekannt sind, für Ihre Bedeutung bei der Progression des Glioblastoms. Außerdem sind pharmakologische Hemmstoffe für diese Ziele verfügbar. In Bezug auf die Signalwege identifizierte die GSEA 21 positiv und 14 negative angereicherte Gensets. Dabei traten der ROS-Signalweg, der mTORC1-Signalweg und der TNFα/NF-κB-Signalweg hervor. Diese stehen in Verbindung mit der ROS-Autophagie-Ferroptose-Achse, was die Beteiligung der ROS-Autophagie-Ferroptose-Achse an der Therapieresistenz bestätigt. Mittels der LEA der Gensets konnten 14 weitere potenzielle *Targets* identifiziert werden, darunter G6PD, SLC7A11, PSMG1, PSMA1 und TXNRD1.

Schlussfolgerung: In der vorliegenden Studie wurde eine umfassende Analyse der molekularen Faktoren, die die Therapieresistenz in menschlichen Glioblastomzellen beeinflussen, durchgeführt. Ziel war es, den Anwendungsbereich gezielter therapeutischer Strategien zu erweitern und die Effektivität und Durchführbarkeit des multi-level integrativen Ansatzes zu validieren. Der umfangreiche Datensatz, der diese Arbeit stützt, ist potenziell wertvoll für weiterführende Forschungen zur Therapieresistenz in Glioblastomzellen. Darüber hinaus kann unser systematischer Arbeitsablauf ohne weiteres auf andere Datensätze und Tumorarten angewendet werden.

5. Publication I

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Integrative analysis of therapy resistance and transcriptomic profiling data in glioblastoma cells identifies sensitization vulnerabilities for combined modality radiochemotherapy

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Abstract

Background: Inherent resistance to radio/chemotherapy is one of the major reasons for early recurrence, treatment failure, and dismal prognosis of glioblastoma. Thus, the identification of resistance driving regulators as prognostic and/or predictive markers as well as potential vulnerabilities for combined modality treatment approaches is of pivotal importance.

Methods: We performed an integrative analysis of treatment resistance and DNA damage response regulator expression in a panel of human glioblastoma cell lines. mRNA expression levels of 38 DNA damage response regulators were analyzed by qRT-PCR. Inherent resistance to radiotherapy (single-shot and fractionated mode) and/or temozolomide treatment was assessed by clonogenic survival assays. Resistance scores were extracted by dimensionality reduction and subjected to correlation analyses with the mRNA expression data. Top-hit candidates with positive correlation coefficients were validated by pharmacological inhibition in clonogenic survival assays and DNA repair analyses via residual γH2AX/53BP1-foci staining.

Results: Inherent resistance to single-shot and similarly also to fractionated radiotherapy showed strong positive correlations with mRNA expression levels of known vulnerabilities of GBM, including PARP1, NBN, and BLM, as well as ATR and LIG4—two so far underestimated targets. Inhibition of ATR by AZD-6738 resulted in robust and dose-dependent radiosensitization of glioblastoma cells, whereas LIG4 inhibition by L189 had no noticeable impact. Resistance against temozolomide showed strong positive correlation with mRNA expression levels of MGMT as to be expected. Interestingly, it also correlated with mRNA expression levels of ATM, suggesting a potential role of ATM in the context of temozolomide resistance in glioblastoma cells. ATM inhibition exhibited slight sensitization effects towards temozolomide treatment in MGMT low expressing glioblastoma cells, thus encouraging further characterization.

Conclusions: Here, we describe a systematic approach integrating clonogenic survival data with mRNA expression data of DNA damage response regulators in human glioblastoma cell lines to identify markers of inherent therapy

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resistance and potential vulnerabilities for targeted sensitization. Our results provide proof-of-concept for the feasibility of this approach, including its limitations. We consider this strategy to be adaptable to other cancer entities as well as other molecular data qualities, and its upscaling potential in terms of model systems and observational data levels deserves further investigation.

Keywords: Glioblastoma, Radiotherapy, Temozolomide, Therapy resistance, Radiosensitization, Chemosensitization, Clonogenic survival, Correlation analysis, DNA damage response, ATR, LIG4, ATM

Background

Radio/chemotherapy is a key treatment option for glioblastoma (GBM), the most common and most aggressive type of primary brain tumor [1–5]. However, GBM is known for its high levels of inherent treatment resistance, giving rise to early recurrence and dismal prognosis with a median survival time of less than 15 months [2, 6, 7]. For patients with recurrent GBM, treatment perspectives are even worse, because therapy resistance has manifested and/or emerged in the course of primary treatment [8, 9]. In order to increase the efficacy of radio/chemotherapy, it is inevitable to identify regulators that drive radio/chemoresistance as potential vulnerabilities for molecularly targeted radio/chemosensitization strategies in combined modality treatment approaches [10, 11].

The DNA damage response (DDR), a conserved network of signaling and DNA repair pathways [12], is of particular interest in this context, since both the expression and the mutational status of DDR genes are frequently altered in malignant cells—especially in GBM [13–15]. Mechanistically, increased and/or accelerated DNA damage repair capacity, enhanced detoxification of reactive oxygen species (ROS), and deficiencies in cell death regulation are frequently involved [16, 17].

Using GBM as a paradigm for a highly treatment-resistant cancer, we developed a two-step integrative approach to identify genes within the DDR network whose expression levels are associated with the degree of radio/chemoresistance in cancer cells. We performed transcriptomic profiling of 38 DDR genes in a panel of seven human GBM cell lines and correlated their expression levels with scores of inherent therapy resistance against ionizing radiation (IR) or temozolomide (TMZ) treatment as extracted by dimensionality reduction of clonogenic survival data. This approach allowed us to identify several DDR genes whose mRNA expression levels showed strong positive correlations with inherent treatment resistance in GBM cells. The druggable top hits were chosen for evaluation in targeted radio/chemosensitization experiments. Proof-of-concept for our approach was provided along several lines, including the confirmation of MGMT mRNA expression as top correlating feature for TMZ resistance and the identification of ATR expression as top correlating feature for radioresistance which was validated by significant radiosensitization of GBM cells upon ATR inhibition in vitro. Nevertheless, not all correlating features could be validated by pharmacological perturbation. Hence, our study provides a blueprint for systematic screens of radio/chemotherapy resistance in preclinical oncology which is evidently transferable to other cancer entities and has promising upscaling potential with regard to the observational data levels.

Methods

Cell lines and reagents

The human GBM cell lines A172, LN18, LN229, and T98G were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and the human GBM cell lines U87-MG, U138-MG, and U251-MG were purchased from Cell Lines Service GmbH (CLS, Eppelheim, Germany). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 0.1 mg/mL streptomycin (all from ThermoScientific, Schwerte, Germany) at 37 °C and 7.5% CO_2. The cell lines were maintained at low passage numbers (< 10 passages) and were regularly tested to be free from mycoplasma (MycoAlert, Lonza; Basel, Switzerland). Cell line identity was confirmed by short tandem repeat (STR) typing (service provided by the DSMZ, Braunschweig, Germany).

The ATR inhibitor AZD-6738 and the ATM inhibitor KU-60019 were both purchased from Absource GmbH (Munich, Germany) and dissolved at 10 mM in dimethylsulfoxide (DMSO, Sigma-Aldrich, Taufkirchen, Germany). The LIG4 inhibitor L189 was purchased from Bio-Techne GmbH (Wiesbaden-Nordenstedt, Germany), and dissolved at 100 mM in DMSO. Temozolomide (TMZ) was purchased from Sigma-Aldrich and dissolved at 100 mM in DMSO. Final concentrations of all drugs tested in the assays were adjusted by directly diluting the respective stock solutions in cell culture medium. Appropriate vehicle dilutions served as controls.

X-ray treatment

Irradiation of cells was performed with an RS-225 X-ray cabinet (X-strahl, Camberley, Great Britain) at 200 kV

and 10 mA (Thoraeus filter, 1 Gy in 243 s) as described [18].

Quantitative real-time PCR (qRT-PCR)

Quantification of mRNA expression levels of DNA damage response (DDR) genes was performed by quantitative real-time RT-PCR (qRT-PCR) as previously described [18–20]. Briefly, total RNA was extracted from cells using the NucleoSpin RNA II extraction kit (Macherey & Nagel, Dueren, Germany). 0.5–1 µg of isolated RNA were mixed with 10 U/µl RevertAid transcriptase, 5 µM Oligo(dT)$_{18}$, 5 µM random hexamers, 1 U/µl Ribolock RNase inhibitor (all from Thermo Scientific), and 500 µM dNTPs (Promega, Heidelberg, Germany) and reversely transcribed. Subsequently, 4 or 20 ng of cDNA were mixed with 300 nM forward and reverse primers in 1× Maxima SYBR Green qPCR Master Mix (ThermoScientific), and subjected to qRT-PCR. qRT-PCR runs were performed with a standard cycling protocol (10 min 95 °C, 45× (15 s 95 °C, 30 s 60 °C)) on an LC480 qPCR cycling platform (Roche Applied Science, Penzberg, Germany). Relative quantification was performed using the ddCT method. Efficiency correction was implemented by using the two different cDNA concentrations (4 and 20 ng cDNA per reaction). Results were normalized to a matrix comprised of three reference genes (18S rRNA, 5'-Aminolevulinate Synthase-1 (ALAS), and β2-Microglobulin (B2M)), and calibrated to the relative expression levels measured for human astrocytes (BioCat, Heidelberg, Germany). Three replicates were analyzed per cell line. Primer sequences have been previously described [19, 20]. Expression values of LN229 and T98G cells had been published before [18] and were re-analyzed for this study.

Determination of MGMT promoter methylation in methylome array

For array-based methylome analysis, DNA was extracted from cells by Qiagen Allprep DNA/RNA mini kit (Qiagen, Hilden, Germany). 500 ng of genomic DNA were subjected to hybridization on Infinium EPIC methylation arrays (Illumina, San Diego, CA; USA), and analyzed according to the manufacturer's instructions. Assays were scanned, and idat files were imported in R using the minfi package and processed according to the Illumina BeadStudio workflow.

Clonogenic survival assay

Clonogenic survival was determined by colony formation assays as described [21, 22]. Briefly, cells were detached with Trypsin/EDTA (ThermoScientific), counted with a Neubauer counting chamber, and up to 55,000 cells per well were seeded into 6-well plates yielding a range of 10–200 colonies after treatment. Cell adherence was allowed for 4 h. For radiation experiments, medium was replaced, cells were irradiated once (single-shot mode) or every 24 h (fractionated mode) with the indicated doses, and incubated at 37 °C and 7.5% CO_2 for up to 12 d to permit colony formation. In case of TMZ treatment, cells were incubated with the indicated TMZ concentrations for 24 h, medium was replaced by TMZ-free medium, and colony formation was allowed for up to 12 d. For combined treatment, cells were additionally irradiated with 0–10 Gy before incubation. For targeted sensitization experiments, the inhibitors were added at the indicated concentrations 30–60 min before IR or TMZ treatment. 24 h later, medium was replaced by inhibitor-free medium.

Colonies were fixed in 80% ethanol and stained with 0.8% methylene blue (both from Merck Millipore, Darmstadt, Germany). All colonies that contained at least 50 cells were counted with a Stemi 305 stereomicroscope (Carl Zeiss, Oberkochen, Germany). Percentages of colony forming cells were calculated and normalized to the respective plating efficiencies at approximately matched colony numbers. Survival curves were subjected to linear-quadratic (for radiation experiments) or logistic (for TMZ treatment) fitting, respectively. At least three independent experiments were performed.

SDS-PAGE and Western Blot

Reducing gradient SDS-PAGE and western blot analyses were performed as previously described [18]. Briefly, cells from cryostocks were thawed, washed with PBS, and lysed with lysis buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% Triton X-100 (v/v, all from Sigma-Aldrich), 1 × EDTA-free protease inhibitor cocktail (Roche)) for 30 min on ice. Protein concentrations were measured by Bradford assay (BioRad, Feldkirchen, Germany). 20 or 400 µg of total protein were subjected to gradient (6–15%) SDS-PAGE, and proteins were transferred onto PVDF Immobilon FL membranes (Merck Millipore). Membranes were blocked with 5% low-fat milk powder (Carl Roth, Karlsruhe, Germany) dissolved in TBST buffer (13 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.02% Triton X-100 (v/v)), and incubated with primary antibodies detecting MGMT (Biozol, Eching, Germany) or vinculin (Sigma-Aldrich) at 4 °C overnight. After washing with TBST, membranes were incubated with IRDye800-conjugated secondary antibodies (LI-COR Biosciences, Bad Homburg, Germany) for 1 h at room temperature. Upon additional washing with TBST, IR800 dye fluorescence was measured with an Odyssey scanner (LI-COR Biosciences).

Immunofluorescence microscopy

Immunofluorescence staining of phosphorylated histone variant H2AX (γH2AX) and p53 binding protein 1 (53BP1) was performed as described [18] and was used to analyze the efficacy of DNA damage repair upon inhibition of identified candidates in terms of residual γH2AX/53BP1-positive DNA damage repair foci 20 h after irradiation. Briefly, 20,000 cells were seeded into 24-well plates supplemented with coverslips and allowed to adhere overnight. Cells were treated with 1.0 μM AZD-6738, 50 μM L189, or DMSO (control) for 1 h before being irradiated at 4 Gy. Cells were fixed 20 h after IR with 3.7% isotonic paraformaldehyde (Merck Millipore) containing 0.1% Triton X-100 (v/v, Sigma-Aldrich) and permeabilized with 0.5% isotonic Triton X-100 (v/v). Unspecific antibody binding was blocked by incubation in 3% isotonic bovine serum albumin (BSA, w/v, Sigma-Aldrich) containing 0.1% Triton X-100 (v/v) for 1 h at room temperature. Cells were stained with monoclonal mouse anti-γH2AX antibody (S139, Merck Millipore) and polyclonal rabbit anti-53BP1 antibody (Bio-Techne), both diluted in 3% isotonic BSA, for 1 h at room temperature. After washing with PBS containing 0.1% Triton X-100 (v/v), cells were stained for 1 h with AlexaFluor488-coupled goat-anti-mouse IgG and AlexaFluor568-coupled goat-anti-rabbit IgG (both from ThermoScientific). DNA was stained with Hoechst 33342 (2.0 μg/ml, Sigma-Aldrich). Upon washing with PBS (0.1% Triton X-100 (v/v)), coverslips were mounted with 4 μl Fluoromount mounting medium (Sigma-Aldrich) onto microscope slides. Microscopic analysis was performed with a Zeiss AxioObserver Z1 inverted microscope equipped with an AxioCam MR Rev3 camera, an LCI Plan-Neofluar 63x/1.3 glycerol objective, and ZEN 2.3 software (all from Carl Zeiss, Oberkochen, Germany). For image acquisition, 31 z-stacks with 250 nm interstack distance were captured, and image deconvolution was performed with the ZEN 2.3 software.

Statistical analysis

Statistical analyses, if not stated otherwise, were performed with OriginPro (OriginLab Ltd., Northhampton, MA, USA). Results are presented as individual data points from individual biological replicates. Group comparisons were performed by two-way ANOVA upon confirmation of normal distribution. Unsupervised hierarchical clustering and principal component analysis (PCA) of z-transformed data were performed as described [19, 21].

Results

mRNA expression levels of DNA damage response (DDR) regulator genes are frequently altered in human GBM cells

The predominant effect of ionizing radiation (IR) and DNA-targeted chemotherapy on cancer cells is the induction of DNA damage of which DNA double-strand breaks are most severe, ultimately leading to cell cycle arrest, cell death, and abrogation of clonogenic survival [23]. Accordingly, resistance against DNA damage-induced cell death and clonogenic inactivation is a major obstacle in current GBM treatment [24], and frequently derives from alterations both in abundance and functionality of DNA damage response (DDR) regulators [25]. In the present study, we examined the basal mRNA expression levels of 38 DDR genes in a panel of seven human GBM cell lines in comparison to primary human astrocytes and observed a thorough and massive upregulation of multiple DDR genes analyzed (Fig. 1A). This is in line with previous reports showing that the abundance of DDR proteins and DDR function are increased in GBM cells [18, 26, 27]. Several of the genes found to be upregulated in our study have been reported to be involved in the recognition of DNA damage and upstream DDR signaling, such as the proximal DDR kinase Ataxia Telangiectasia and RAD3 Related (ATR), the MRN complex components Meiotic Recombination 11 Homolog A (MRE11A), RAD50, and Nibrin (NBN) [28], or the single strand DNA-binding Replication Protein A1 (RPA1), respectively. Other genes found to be upregulated included Flap-structure-specific Endonuclease 1 (FEN1), Exonuclease 1 (EXO1), Heat Shock Proteins 90AA1 and 90AB1 (HSP90AA1 and HSP90AB1), and members of the X-ray Repair Cross-Complementing (XRCC) family.

We also detected some genes whose mRNA expression levels were downregulated in GBM cells as compared to normal healthy astrocytes (Fig. 1A). Among these genes, several candidates with documented tumor-suppressive functions were found, such as TP53, and CDKN1A [29, 30]. Interestingly also, the gene encoding for O^6-Methylguanine-DNA-methyltransferase (MGMT), a DNA repair enzyme known to remove alkylating modifications from DNA thereby counteracting the antitumorigenic effect of temozolomide (TMZ) [31], was in principal downregulated in GBM cells as compared to normal astrocytes, and in several GBM cell lines it was not detectable at all, both on mRNA and protein level (Fig. 1A and C).

To analyze this aspect in further detail, we also determined the methylation status of the MGMT promoter in our GBM cell line panel by methylome arrays (Fig. 1B). As expected, MGMT promoter methylation and mRNA expression levels showed an inverse relation, and the mRNA expression levels were in good accordance with

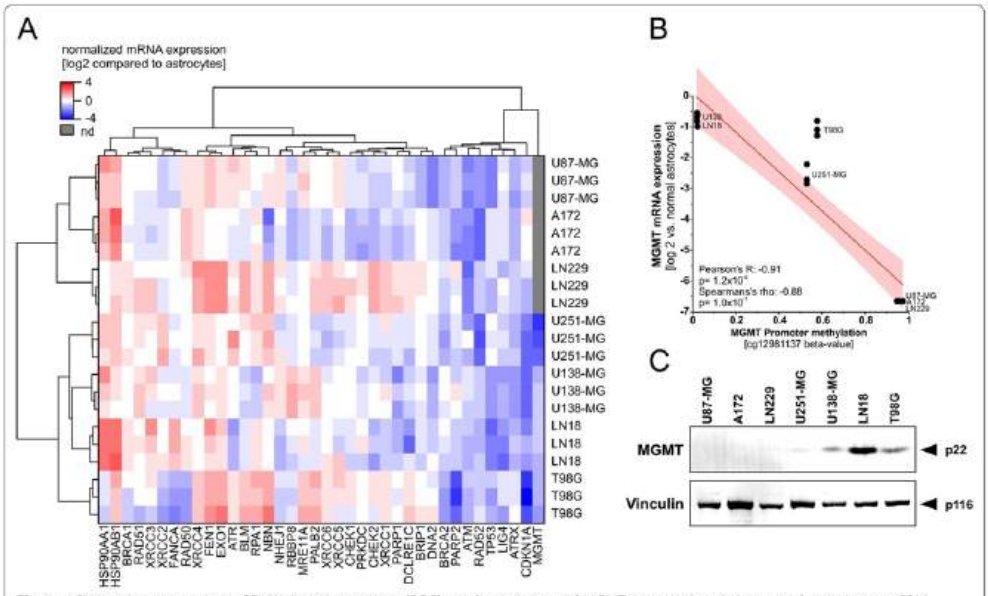

Fig. 1 mRNA expression analysis of DNA damage response (DDR) regulator genes, and MGMT promoter methylation analysis in human GBM cell lines. **A** Basal mRNA expression levels of DDR regulator genes in GBM cell lines A172, LN18, LN229, T98G, U87-MG, U138-MG, and U251-MG as measured by qRT-PCR (ddCT method). Expression levels were normalized to a matrix of three reference genes (18S rRNA, β2-Microglobulin, and 5'-Aminolevulinate Synthase-1), and calibrated on the expression levels of astrocytes. Three replicates were analyzed per cell line. Expression values (log2-transformed) and samples were subjected to unsupervised hierarchical clustering. **B** Correlation of MGMT promoter methylation status and MGMT mRNA expression levels in the employed GBM cell lines as detected by methylome array and qRT-PCR, respectively. **C** Protein expression levels of MGMT in GBM cells as measured by western blot. Vinculin served as loading control

the observed protein expression levels (Fig. 1A and B). In summary, our DDR transcriptomic profiling screen revealed that basal mRNA expression levels of DDR genes are frequently altered in human GBM cells, suggesting potential vulnerabilities for targeted inhibition and sensitization strategies in combined modality treatment approaches.

Human GBM cell lines exhibit different levels of inherent resistance towards IR, TMZ or the combination thereof in clonogenic survival assays

To identify differences in inherent resistance towards IR and TMZ across our GBM cell line panel, we performed clonogenic survival assays. In analogy to our previous studies with other cancer entities, clonogenic survival data were subjected to dimensionality reduction via principal component analysis (PCA), and the extracted scores of the first principal component (PC1) were used as a measure of treatment resistance [19–21] (Fig. 2). We observed marked differences in clonogenic survival after single-shot IR as well as after fractionated IR (5 × 2 Gy). U251-MG, T98G, and U87-MG revealed highest levels of inherent resistance to single-shot IR, while LN18, U138-MG, and LN229 were most sensitive. Similarly, resistance to fractionated IR was highest in U251-MG, and T98G cells, while U138-MG and LN229 again showed highest sensitivity. Resistance to TMZ treatment, on the contrary, was highest in LN18, T98G, and U138-MG cells, the cell lines with the lowest levels of MGMT promoter methylation (Fig. 1B) and highest levels of MGMT mRNA expression (Fig. 1A). The combined treatment comprising single-shot IR and TMZ displayed a similar resistance pattern as the single-shot IR only treatment (Fig. 2). Again, highest levels of resistance were observed in U251-MG, T98G, and U87-MG cells, while U138-MG, and LN229 were most sensitive. However, the additive effect of both treatment modalities was clearly observed exemplarily at the LN18 cell line which showed highest sensitivity to single-shot IR mono-treatment, and highest resistance to TMZ mono-treatment, resulting in an intermediate resistance score for the combination treatment.

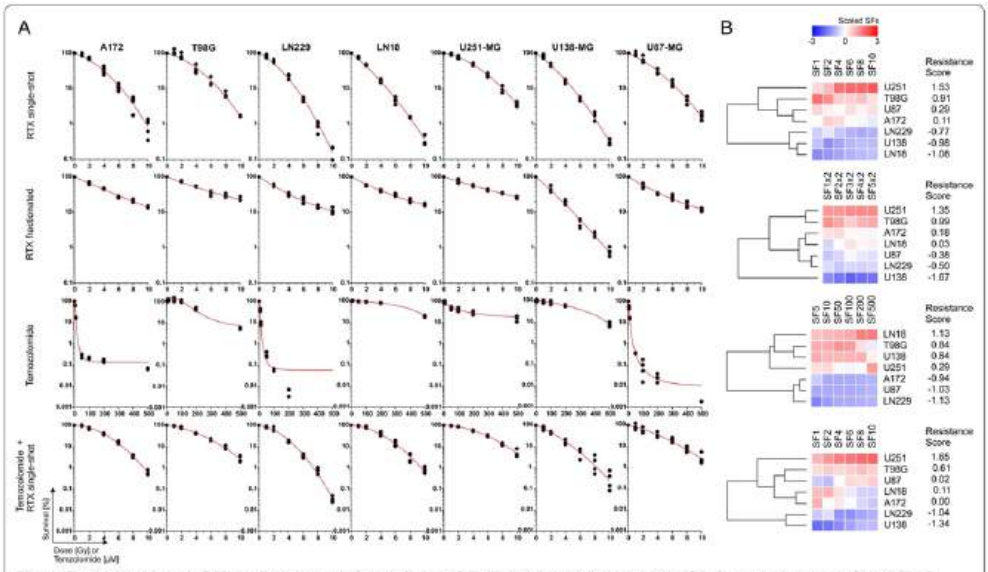

Fig. 2 Clonogenic survival of GBM cells upon treatment with X-ray IR, TMZ, and the combination thereof. **A** Clonogenic survival of A172, T98G, LN229, LN18, U251-MG, M138-MG, and U87-MG cells after single-shot IR (0–10 Gy), fractionated IR (0–5 fractions of 2 Gy each), TMZ treatment (0–500 µM for 24 h), and combination of 5 µM TMZ (24 h) and single-shot IR (0–10 Gy) as determined by colony formation assays. Results of at least three independent experiments are depicted. Super-imposed fitting functions are linear-quadratic in case of radiation experiments and logistic in case of TMZ treatment. **B** Z-transformed survival data are depicted as unsupervised hierarchical clustering. Principal component analysis (PCA)-derived resistance scores (scores of PC1 as described in [21]) are shown

Correlation of therapy resistance data with transcriptomic profiling data identifies ATR, LIG4, and ATM as potential candidates for targeted sensitization of GBM cells

In order to identify markers and potential drivers of inherent resistance against IR and TMZ in GBM cells, we combined the data from our qRT-PCR and radio/chemoresistance screens (Figs. 1A and 2) and performed Spearman correlation analyses of the therapy resistance scores (PC1s) with the mRNA expression levels of the 38 DDR genes (Fig. 3). For IR applied in single-shot mode, we observed the highest positive correlation for ATR, suggesting an involvement of ATR in GBM radioresistance. We also obtained positive correlations for TP53, RPA1, LIG4, NBN, BLM, and PARP1 offering interesting opportunities for molecularly targeted radiosensitization approaches. Highly sophisticated inhibitors are available for some of these candidates, including ATR and PARP1 [32–34]. For LIG4, first-line inhibitors, such as L189, have recently been developed [35], and the MRN complex comprising NBN, and its satellite BLM has been shown to be vulnerable for instance to inhibition of HSP90 chaperoning activity [36]. The radiosensitizing effects of PARP1 and HSP90 inhibition on GBM cells have been shown by many groups including ours [18, 37–39], thus providing principal validating evidence for our correlation approach and confirming the functional involvement of the potential target genes identified. ATR and LIG4 were picked as novel targets in GBM, and so we decided to examine the effects of ATR and LIG4 inhibition on radiosensitization of GBM cells in greater detail. Correlation of the fractionated radioresistance scores with the transcriptomic data revealed in principal similar trends, yet with slightly different ranking. Here, RPA1, ATRX, and HSP90AB1 were the top positively correlating hits (Fig. 3).

Resistance against TMZ showed the highest positive correlation with the expression levels of MGMT, once more confirming the feasibility of our methodological approach (Fig. 3). However, it also showed a strong positive correlation with expression of ATM, the major upstream signaling kinase of the DDR network [40] (Fig. 3). This finding was rather unexpected, since it suggested that ATM might play a hitherto unknown role in TMZ resistance of GBM cells. Therefore, we decided to test this hypothesis by interfering with ATM kinase activity in combination with TMZ treatment [41].

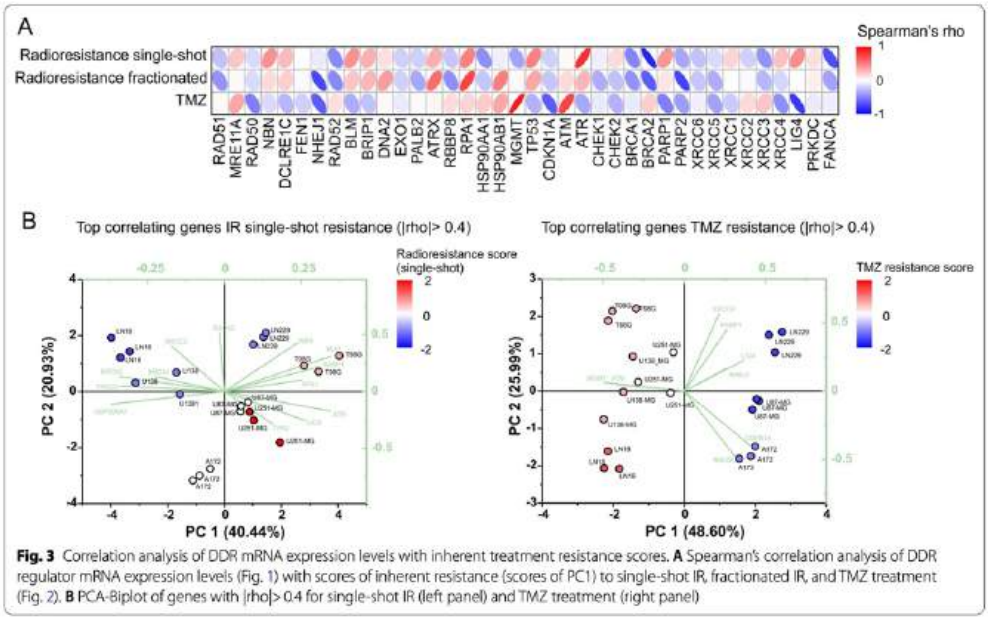

Fig. 3 Correlation analysis of DDR mRNA expression levels with inherent treatment resistance scores. **A** Spearman's correlation analysis of DDR regulator mRNA expression levels (Fig. 1) with scores of inherent resistance (scores of PC1) to single-shot IR, fractionated IR, and TMZ treatment (Fig. 2). **B** PCA-Biplot of genes with |rho|> 0.4 for single-shot IR (left panel) and TMZ treatment (right panel)

Functional validation confirms inhibition of ATR but not LIG4 as a strategy to increase the efficacy of radiotherapy in GBM

We proceeded to validate our candidates by pharmacological interference with their functions. Deliberately, we concentrated on ATR and LIG4 in terms of sensitization towards single-shot IR and on ATM in terms of sensitization towards TMZ treatment. The other candidates were left out either because of their undruggability (e.g. RPA1, ATRX), or because of already existing evidence for their performance as sensitization targets in GBM (PARP1 and MRN complex) [36–38, 42, 43].

For ATR inhibition, we made use of the highly brain-penetrable ATR inhibitor AZD-6738 [44, 45]. As cell models, one GBM cell line of moderate radioresistance (A172) and one cell line of high radioresistance (U251-MG) were chosen (Fig. 2). Inhibitor treatment was performed for 24 h, starting 1 h before irradiation. Afterwards, medium was replaced and colony formation was allowed in inhibitor-free medium. Both cell lines were convincingly sensitized to single-shot IR treatment by AZD-6738 in a dose-dependent manner (Fig. 4A), confirming that ATR indeed could serve as a target for radiosensitization in GBM [46]. This was also supported by analyses of residual DNA damage upon irradiation as detected by immunofluorescence staining of phosphorylated histone variant H2AX (γH2AX) and 53BP1, two well-established markers of DNA damage repair foci [47, 48]. We observed an accumulation of residual γH2AX/53BP1-foci 20 h after single-shot irradiation at 4 Gy in the presence of AZD-6738. Additionally, the combination treatment resulted in a massive increase in micronuclei formation in both cell lines analyzed (Fig. 4A), indicating a reduction in DNA damage repair efficacy in GBM cells upon ATR inhibition which should be further investigated in in vivo models. We next interrogated whether inhibition of LIG4 could also sensitize GBM cells to IR. LIG4 is the major DNA ligase of the non-homologous end joining (NHEJ) pathway [49], rendering it a promising target for sensitization attempts. Inhibition experiments were performed in analogy to the procedure described for ATR inhibition. However, inhibition of LIG4 activity by the poly-DNA-ligase inhibitor L189 [35] exhibited no obvious effects on radioresistance of GBM cells (Fig. 4B), at least in the cell lines tested (A172, U251-MG). We also did not observe marked differences in residual γH2AX/53BP1 repair foci, nor in the formation of micronuclei (Fig. 4B), again confirming that L189 had no obvious effects on the response of A172 and U251-MG cells to ionizing irradiation. Technically, we cannot rule out that this derives from the suboptimal

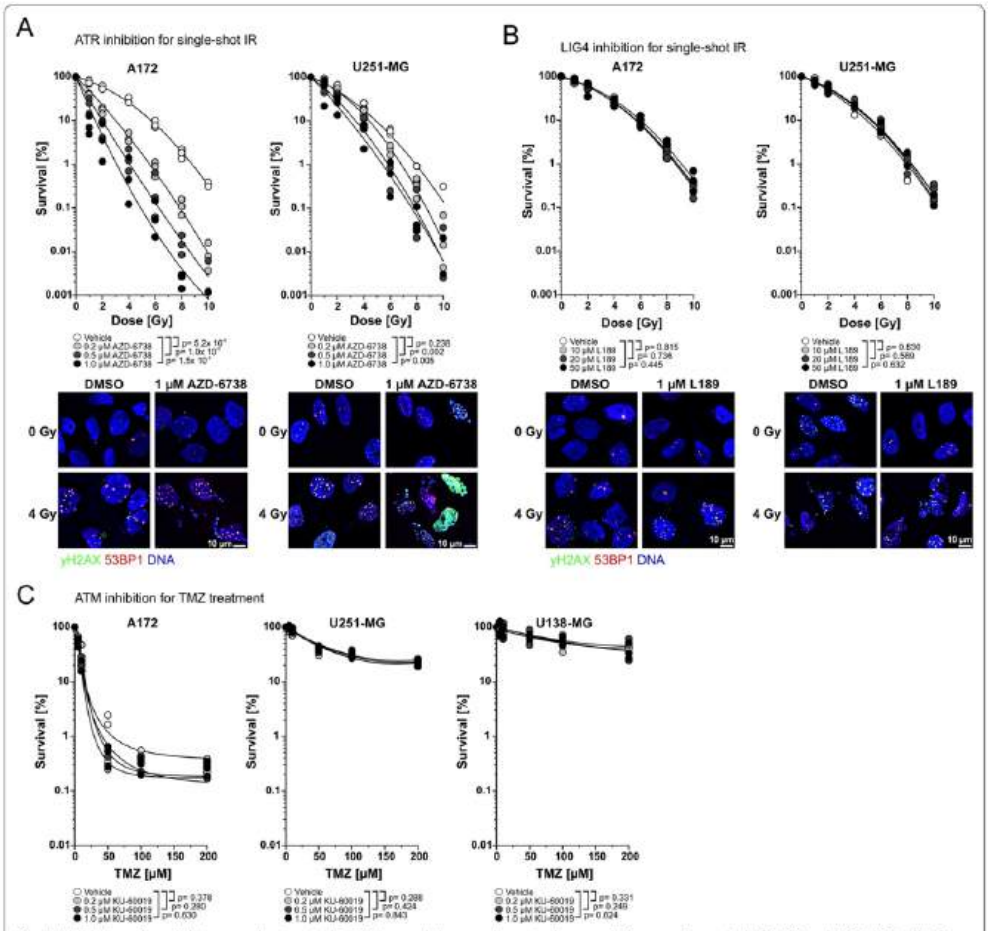

Fig. 4 Validation of candidate genes by targeted inhibition and clonogenic survival assays. **A** Clonogenic survival of A172 and U251-MG cells after single-shot IR (0–10 Gy) and ATR inhibition by AZD-6738 (0–1.0 µM, upper panel). Immunofluorescence staining of γH2AX and 53BP1 20 h after irradiation at 4 Gy ± ATR inhibition by 1 µM AZD-6738 (lower panel). γH2AX is depicted in green, 53BP1 in red, and DNA is depicted in blue. Scale bar represents 10 µm. **B** Clonogenic survival of A172 and U251-MG cells after single-shot IR and LIG4 inhibition by L189 (0–50 µM, upper panel). Immunofluorescence staining of γH2AX and 53BP1 20 h after irradiation at 4 Gy ± LIG4 inhibition by 50 µM L189 as performed in A (lower panel). **C** Clonogenic survival of A172, U138-MG, and U251-MG cells after 24 h treatment with TMZ (0–200 µM) and ATM inhibition by KU-60019 (0–1.0 µM). Super-imposed fitting functions are linear-quadratic in case of radiation experiments and logistic in case of TMZ treatment. Results of three independent experiments are depicted for each condition, and p-values were calculated by two-way ANOVAs

affinity and/or specificity of the L189 inhibitor for LIG4 [50]. Furthermore, it is feasible to assume that DNA ligases other than LIG4 are overexpressed in GBM cell lines and thereby compete with LIG4 for inhibitor binding [50]. Thus, we recommend the re-evaluation of LIG4 as a candidate for targeted radiosensitization of GBM as soon as inhibitors with improved pharmacological profile are available in order to clarify if LIG4 is mechanistically involved in GBM radioresistance or if the correlation of its expression levels with inherent radioresistance has no causal implications.

Functional validation reveals ATM inhibition as a strategy to increase the sensitivity of GBM cells to TMZ treatment to be complex

The unexpected strong correlation between inherent resistance of GBM cells to TMZ and mRNA expression levels of ATM prompted us to investigate whether inhibition of ATM could indeed increase the efficacy of TMZ treatment in resistant GBM cells. To this end, we employed the ATM inhibitor KU-60019 [41]. Inhibitor treatment of GBM cells with different MGMT promoter methylation status and mRNA/protein expression levels (Fig. 1) was performed 1 h prior to the addition of TMZ for 24 h. Then, medium was replaced, and colony formation was allowed in inhibitor-free medium (Fig. 4C). KU-60019 treatment had no obvious effects on TMZ sensitivity of GBM cells negative (U138-MG) or moderately positive (U251-MG) for MGMT promoter methylation, and accordingly positive for MGMT mRNA expression (Fig. 1)—even though the expression levels in these cell lines were lower than in normal astrocytes (Fig. 1A). Nevertheless, slight but statistically not significant sensitizing effects towards TMZ by KU-60019 were observed for A172 cells (Fig. 4C), a cell line positive for MGMT promoter methylation and very low MGMT mRNA and protein expression levels (Fig. 1). Similar findings for an involvement of ATM in TMZ resistance of MGMT low expressing GBM cells have been also reported by others [51–53]. Thus, it needs to be further investigated if ATM inhibition may represent a sensitization approach for TMZ treatment of GBM with low MGMT expression.

Collectively, our results show that correlating clonogenic survival data with mRNA expression data followed by functional validation in form of pharmacological perturbation represents a feasible approach to identify markers and potential vulnerabilities of radio/chemotherapy resistance and that the major targets identified by this approach deserve further characterization.

Discussion

Here, we describe a systematic, integrative approach for the analysis of inherent treatment resistance of GBM cells. It is based upon the integration of clonogenic survival data with mRNA expression data and aims at the identification of regulators involved in inherent treatment resistance which represent interesting targets for sensitizing strategies in combined modality radio/chemotherapy. We chose a cell line panel of GBM as a model of an inherently highly treatment-resistant cancer entity [2] and the DNA damage response (DDR) as a compilation of genes with high relevance for resistance against radiotherapy and/or DNA targeting chemotherapy [54].

Our correlative analyses confirmed markers of GBM treatment resistance that are already well-known, such as MGMT expression in the context of TMZ resistance. It also disclosed potential targets for pharmacological sensitization that have previously been validated, such as PARP1 and HSP90 with regard to resistance against radiotherapy [18, 37–39, 55]. These findings serve as proof-of-concept for our strategy. Furthermore, our screen identified additional, so far underestimated candidates for targeted sensitization, including ATR in the context of GBM radioresistance. Since functional validation of ATR's involvement in GBM radioresistance with the ATR inhibitor AZD-6738 provided very convincing radiosensitization of GBM cells in vitro, and similar evidence has also been provided by others [46], ATR inhibition in combined modality GBM radiotherapy certainly deserves more in-depth preclinical investigation. Similarly, the association of ATM expression with TMZ resistance in the context of MGMT low expressing GBM is an interesting novel finding disclosed by our study. Since similar findings were published before [51–53], and ATM inhibitors are currently undergoing clinical trialing in GBM (NCT03423628, NCT05182905), this observation should be further investigated in future. Nevertheless, there were also genes whose expression levels correlated with therapy resistance and whose functional involvement could not be validated. As such, inhibition of LIG4 did not noticeably sensitize GBM cells towards radiotherapy. This may derive from the correlative, not causative nature of our screen [56] as well as from the suboptimal pharmacological characteristics of the used inhibitor L189 [50]. So, the performance of LIG4 targeting in combined modality GBM radiotherapy should be preclinically re-evaluated as soon as improved next generation LIG4 inhibitors are available.

Overall, our findings demonstrate the feasibility as well as the limitations of this correlative approach for the identification of markers of inherent radio/chemoresistance and potential targets for pharmacological sensitization. This is not restricted to glioblastoma, and instead may be adapted to other cancer entities as well [19]. It is also not limited to targeted expression analyses of small gene sets, such as the DDR selected for our study. In fact, we consider it to be well-applicable to larger gene sets, whole transcriptome data, and other OMICs levels. Very recently, we also applied it to functional datasets of cell fate decisions and identified the early induction of tumor cell senescence in conjunction with the production of senescence-associated cytokines as therapeutically targetable determinants of radioresistance in head-and-neck squamous cell carcinoma (HNSCC) [20].

Conclusions

The persistent limitation in treatment success of radio/chemotherapy in highly resistant cancer entities such as GBM demands for the identification of novel markers that contribute to therapy resistance and represent targets for pharmacological interference. Here, we present a systematic, integrative approach correlating clonogenic survival data with mRNA expression data to identify markers and drivers of radio/chemotherapy resistance in GBM. Our findings provide proof-of-concept for the feasibility of this approach as well as its limitations and open the perspective to apply it to other cancer entities and large-scale molecular data sets.

Abbreviations

53BP1: P53 binding protein 1; ALAS: 5'-Aminolevulinate synthase-1; ATM: Ataxia telangiectasia mutated; ATR: Ataxia telangiectasia and RAD3 related; ATRX: Alpha thalassemia/mental retardation syndrome X-linked; B2M: β2-Microglobulin; BLM: Bloom syndrome RecQ like helicase; BSA: Bovine serum albumin; CDKN1A: Cyclin-dependent kinase inhibitor 1A; DDR: DNA damage response; DMEM: Dulbecco's modified eagle medium; DMSO: Dimethylsulfoxide; EDTA: Ethylene diamine tetraacetic acid; EXO1: Exonuclease 1; FCS: Fetal calf serum; FEN1: Flap-structure specific endonuclease 1; γH2AX: Phosphorylated histone variant H2AX; GBM: Glioblastoma; HNSCC: Head-and-neck squamous cell carcinoma; HSP90AA1: Heat shock protein 90 AA1; HSP90AB1: Heat shock protein 90 AB1; IR: Ionizing radiation; LIG4: DNA Ligase 4; MGMT: O^6-Methylguanine-DNA-methyltransferase; MRE11A: Meiotic recombination 11 homolog A; MRN: MRE11-RAD50-NBN; NBN: Nibrin; NHEJ: Non-homologous end-joining; PBS: Phosphate-buffered saline; PC1: Principal component 1; PCA: Principal component analysis; qRT-PCR: Quantitative realtime polymerase chain reaction; RAD50: RAD50 double strand break repair protein; RPA1: Replication protein A1; STR: Short tandem repeat; TMZ: Temozolomide; TP53: Tumor-suppressor protein 53; XRCC: X-ray repair cross-complementing.

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Author contributions

LES, VA, NB, AEN, DFF, MN, JH, CB, KU, KL, and MO conceived the experiments, analyzed the data, and discussed the data. LES, VA, NB, AEN, and MO performed the experiments. KL and LES prepared the figures and performed the statistical analyses. MO, KL and LES wrote the manuscript. All authors revised the manuscript and agreed to its final version.

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Availability of data and materials

The data presented in this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

All authors declare that there is no conflict of interest.

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Systematic in vitro analysis of therapy resistance in glioblastoma cell lines by integration of clonogenic survival data with multi-level molecular data

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Abstract

Despite intensive basic scientific, translational, and clinical efforts in the last decades, glioblastoma remains a devastating disease with a highly dismal prognosis. Apart from the implementation of temozolomide into the clinical routine, novel treatment approaches have largely failed, emphasizing the need for systematic examination of glioblastoma therapy resistance in order to identify major drivers and thus, potential vulnerabilities for therapeutic intervention. Recently, we provided proof-of-concept for the systematic identification of combined modality radiochemotherapy treatment vulnerabilities via integration of clonogenic survival data upon radio(chemo)therapy with low-density transcriptomic profiling data in a panel of established human glioblastoma cell lines. Here, we expand this approach to multiple molecular levels, including genomic copy number, spectral karyotyping, DNA methylation, and transcriptome data. Correlation of transcriptome data with inherent therapy resistance on the single gene level yielded several candidates that were so far underappreciated in this context and for which clinically approved drugs are readily available, such as the androgen receptor (AR). Gene set enrichment analyses confirmed these results, and identified additional gene sets, including reactive oxygen species detoxification, mammalian target of rapamycin complex 1 (MTORC1) signaling, and ferroptosis/autophagy-related regulatory circuits to be associated with inherent therapy resistance in glioblastoma cells. To identify pharmacologically accessible genes within those gene sets, leading edge analyses were performed yielding candidates with functions in thioredoxin/peroxiredoxin metabolism, glutathione synthesis, chaperoning of proteins, prolyl hydroxylation, proteasome function, and DNA synthesis/repair. Our study thus confirms previously nominated targets for mechanism-based multi-modal glioblastoma therapy, provides proof-of-concept for this workflow of multi-level data integration, and identifies novel candidates for which pharmacological inhibitors are readily available and whose targeting in combination with radio(chemo)therapy deserves further examination. In addition, our study also reveals that the presented workflow requires mRNA expression data, rather

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than genomic copy number or DNA methylation data, since no stringent correlation between these data levels could be observed. Finally, the data sets generated in the present study, including functional and multi-level molecular data of commonly used glioblastoma cell lines, represent a valuable toolbox for other researchers in the field of glioblastoma therapy resistance.

Keywords Glioblastoma, Therapy resistance, Multi-level molecular data, Correlation analysis

Introduction

Glioblastoma, despite immense efforts in preclinical, translational, and clinical research during the last decades still remains a daunting disease with highly dismal prognosis [1]. Fractionated radio(chemo)therapy with 30 fractions of 2 Gy and concomitant administration of DNA-alkylating temozolomide (TMZ) in definitive or adjuvant settings, followed by TMZ-based maintenance therapy remains the standard of care [2, 3]. However, glioblastoma is well-known for its high degree of inherent therapy resistance, both to ionizing radiation (IR) and TMZ, resulting in frequent treatment failure and early recurrence [4]. Thus, a more detailed understanding of the biological and molecular mechanisms underlying glioblastoma therapy resistance is needed in order to open new perspectives for molecularly targeted therapy and to improve disease prognosis [5, 6].

Based on our recent proof-of-concept study [7], we here present a systematic approach which integrates inherent resistance data of commonly used human glioblastoma cell lines to IR and TMZ with multi-level molecular data of those cell lines obtained under treatment-naive conditions, including spectral karyotyping (SKY FISH), array comparative genomic hybridization (aCGH), array-based DNA methylation, and transcriptomic (mRNA microarray) analyses. By integrating these data with scores of inherent therapy resistance as extracted by dimensionality reduction of clonogenic survival data ([7, 8], Additional file 1: Table S1), and subsequent gene set enrichment analysis (GSEA) [9], we could identify several candidate networks and signaling circuits which are associated with inherent treatment resistance of glioblastoma cells and can be readily targeted by clinically approved or at least clinically trialed drugs. Among these were pathways involved in homeostasis of reactive oxygen species (ROS), mammalian target of rapamycin complex 1 (mTORC1) signaling, and androgen receptor (AR) signaling [10, 11]. Taken together, we present a novel integrative approach for the systematic identification of therapeutic vulnerabilities (not only) in glioblastoma as well as potential candidates whose targeting in conjunction with radiotherapy and/or TMZ may help to break glioblastoma therapy resistance.

Methods

Cell culture

The human glioblastoma cell lines A172, LN18, LN229, T98G, U87 [12], U138, and U251 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), or from Cell Lines Service GmbH (CLS, Eppelheim, Germany), and confirmed for identity by short tandem repeat (STR) typing according to the standards of the ATCC and the American National Standards Institute (ANSI, New York, NY, USA) of 2011 (ANSI/ATCC ASN-0002–2011) [13]. All cell lines were cultured in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 0.1 mg/mL streptomycin (all from Thermo Scientific, Schwerte, Germany) at 37 °C and 7.5% CO_2. Cells were kept at low passage numbers ($\leq$ 10 passages), and were regularly tested by MycoAlert assay (Lonza, Basel, Switzerland) to be free of mycoplasma contamination.

Determination of therapy resistance scores

Resistance to therapy was determined by clonogenic survival assays as described [7]. In brief, cells were seeded into 6-well plates and incubated for 4 h in order to adhere. Cells were irradiated at the indicated doses in single-shot or in intervals of 24 h (fractionated mode), and colony formation was allowed for up to 12 d. In case of TMZ treatment, cells were incubated with TMZ at the indicated doses for 24 h, medium was changed, and colony formation was performed in TMZ-free medium. For the combination treatment, cells were exposed to 5 µM TMZ for 24 h, irradiated at the indicated doses, and incubated in TMZ-free medium for colony formation. Colonies were fixed with 80% ethanol, stained with 0.8% methylene blue (both from Merck Millipore, Darmstadt, Germany), and counted with a Stemi 305 stereomicroscope (Carl Zeiss, Oberkochen, Germany) as described [7, 14]. Percentages of colony forming cells were calculated and normalized to the respective plating efficiencies at approximately matched colony numbers. Resistance scores were extracted by principal component analysis (PCA) as scores of the first principal component (PC1) [8].

Spectral karyotyping (SKY FISH)

For spectral karyotyping (SKY FISH) analyses, cells at 80% confluency were accumulated in M-phase by treatment with 0.1 µg/ml colcemid (Roche Diagnostics, Basel, Switzerland) for 3 h. Cells were then harvested with TrypLE Express (Thermo Scientific), and incubated in 4.0% (w/v) potassium chloride for 45 min at 37 °C. Cells were fixed with fixative (methanol and glacial acetic acid at 3:1 ratio, both from Merck Millipore) for 45 min at 4 °C, washed, and resuspended in fixative. Cell suspensions were dripped onto microscope slides, and hybridization was performed using the denatured SKY-probe mixture kit SkyPAINT DNA (Applied Spectral Imaging, Carlsbad, CA, USA) as previously described [15]. For staining, slides were incubated with anti-digoxigenin (Roche), avidin-Cy5, and avidin-Cy5.5 antibodies (Biomol, Hamburg, Germany), and counterstained with 0.1% (w/v) 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Taufkirchen, Germany). Slides were supplemented with Vectashield mounting solution (Vector Laboratories, Burlingame, CA, USA), and spectral imaging was performed with a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a SpectraCube device and SkyView software (both from Applied Spectral Imaging). Description of karyotypes was performed according to the international system of human cytogenetic nomenclature (ISCN, edition 2013) [16].

Global gene expression microarrays

To analyze global mRNA expression levels in glioblastoma cells, gene expression microarray analysis was performed. In brief, total RNA was extracted from cells using the Qiagen Allprep DNA/RNA mini kit (Qiagen). RNA concentration was determined with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific), and RNA quality was assessed by a total RNA 6000 nano chip assay performed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). To obtain global mRNA expression data, 50 ng of total RNA was subjected to Agilent SurePrint G3 human 8 × 60 k V2 microarray analysis (AMADID 039,494, Agilent Technologies). RNA from untransformed human astrocytes (Provitro AG, Berlin, Germany) served as reference. Results were collected using the Agilent feature extraction software (version 10.7, Agilent Technologies), and exported as text files. Assessment of data quality, filtering, and data processing were performed with the Bioconductor R packages Limma and Agi4 × 44PreProcess as previously described [17], and data analysis was performed with R. The obtained data are available at Gene Expression Omnibus (GEO, super set accession number: GSE119637) and under the link https://www.ncbi.nih.gov/geo/query/acc.cgi?acc=GSE119637 using the token 'sfqnocumrvofrmv'.

Array comparative genomic hybridization (aCGH)

To identify genomic copy number alterations (CNAs) in glioblastoma cells, array comparative genomic hybridization (aCGH) analyses were performed as previously described [18]. In brief, DNA was extracted from cells by Qiagen Allprep DNA/RNA mini kit (Qiagen), concentration and quality of DNA were assessed with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific), and 120 ng DNA was fluorescently labelled using the CYTAG SuperCGH labelling kit (Enzo Life Sciences, New York, NY, USA). After removing free nucleotides using Microcon YM-30 columns (Merck Millipore), labelled DNA was subjected to oligonucleotide-based high-resolution SurePrint G3 Human 60 k CGH microarray analysis (AMADID 021,924, Agilent Technologies). Microarrays were scanned with a G2505C SureScan microarray scanning system (Agilent Technologies), raw data were extracted using the Agilent feature extraction software (version 10.7, Agilent Technologies), and CNA regions were identified using the Bioconductor R packages CGHcall and CGHregions [18, 19]. A compilation of all molecular data collected in this study is deposited as an Excel file in the Additional file 2.

Array-based DNA methylation analyses

Array-based DNA methylation analyses were performed as previously described [7]. In brief, DNA was extracted by Qiagen Allprep DNA/RNA mini kit (Qiagen), concentration and quality of DNA were assessed with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific), and 500 ng DNA was subjected to hybridization on an Infinium EPIC methylation array (Illumina, San Diego, CA; USA). The arrays were scanned, idat files were imported in R using the minfi package [20], and processed in accordance to the Illumina BeadStudio data analysis workflow (Illumina). Beta values were used for downstream hierarchical clustering analysis along with the beta values retrieved for glioblastoma samples from TCGA database https://www.cancer.gov/tcga [21, 22].

Comparison of transcriptome and methylome profiles of glioblastoma cell lines with those from glioblastoma and lower-grade glioma (LGG) patient samples

Transcriptome profiles and methylome beta values from glioblastoma and LGG patient samples were retrieved from TCGA (https://tcga-data.nci.nih.gov/docs/publications/lgggbm_2015/) [21, 22]. Transcriptome profiles

of 560 glioblastoma and 463 LGG patients were corrected for putative batch effects and z-scaled per gene before combination with the transcriptomic data obtained from the cell lines in a common gene expression matrix. The top 20 differently expressed genes between glioblastoma and LGG samples were analyzed by hierarchical clustering (Fig. 1b, clustering method ward.D and euclidean distance measure), and principal component analysis (PCA).

For DNA methylation, the profiles of 410 glioblastoma and 516 LGG patients were included, and a merged beta value matrix including the methylation profiles of glioblastoma and LGG patients as well as those of the glioblastoma cell lines matching the CpG island methylator phenotype (CIMP) signature by Ceccarelli et al. [21] was generated. The methylation profiles were subjected to hierarchical clustering analysis (Fig. 1c, clustering method ward.D and euclidean distance measure), and analyzed by PCA.

Molecular subtyping based on cytogenetic and transcriptomic data

Molecular subtyping was performed via different approaches. For calculation of molecular subtype scores (Additional file 1: Table S3), the data derived from cytogenetic characterization (SKY FISH) and aCGH analyses together with the expression data of relevant driver genes were utilized. All molecular subtype features of a cell line

A

	A172	LN18	T98G	U138	U87	LN229	U251
RRID	CVCL_0131	CVCL_0392	CVCL_0556	CVCL_0020	CVCL_0022	CVCL_0393	CVCL_0021
Sex	male	male	male	male	male	female	male
Age	53 years	61 years	61 years	47 years	unspecified	60 years	75 years
Population	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
IDH1	wildtype	wildtype	wildtype	wildtype	wildtype	wildtype	wildtype
P53		mutant (C238S)	mutant (M237I)	mutant (R213Q)		mutant (P98L)	mutant (R273H)
MGMT promoter methylation	positive	negative	weak	negative	positive	positive	weak
MGMT mRNA expression	negative	positive	positive	positive	negative	negative	weak

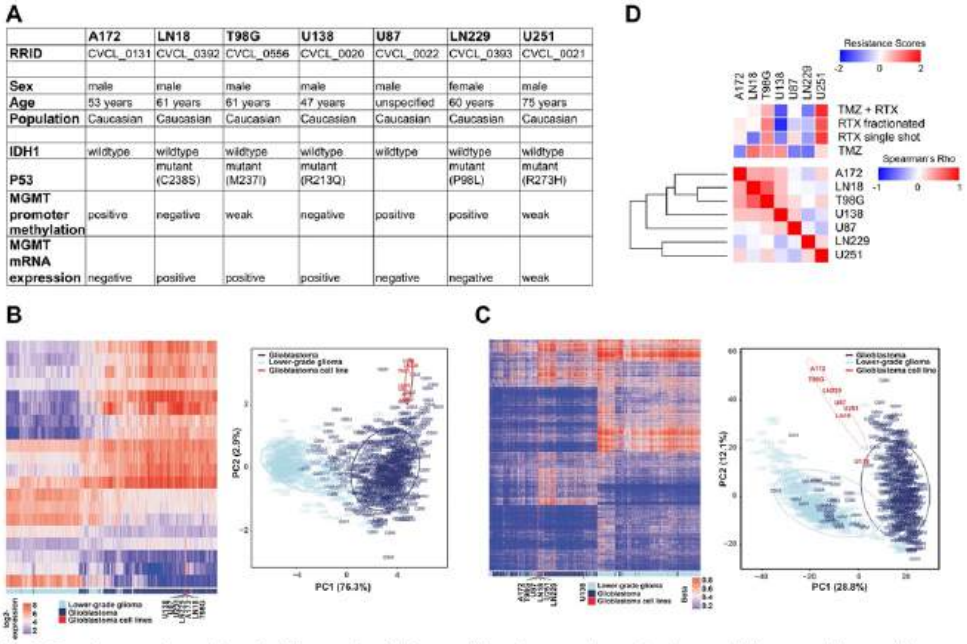

Fig. 1 Sample-to-sample correlation of mRNA expression of 100 most differently expressed genes in a human glioblastoma cell line panel does not match with inherent therapy resistance **a** Tabular presentation of characteristics of the human glioblastoma cell lines as obtained from the Cellosaurus database (https://web.expasy.org/cellosaurus/). **b** Unsupervised hierarchical clustering and principal component analysis (PCA) of mRNA expression levels of top 20 genes differently expressed between glioblastoma and low-grade glioma (LGG) patient samples (data from the TCGA database (https://tcga-data.nci.nih.gov/docs/publications/lgggbm_2015/), clustering method ward.D and euclidean distance measure) in 560 glioblastoma and 463 LGG patient samples, and in glioblastoma cell lines. **c** Unsupervised hierarchical clustering and PCA of G-CIMP signatures for hypermethylation phenotypes in 410 glioblastoma and 516 LGG patient samples (data from the TCGA database), and in glioblastoma cell lines. **d** Sample-to-sample correlation analysis of mRNA expression of 100 genes with highest intra-panel variation in expression in human glioblastoma cell lines. Expression values were determined by global gene expression microarray analysis. PCA-derived scores of inherent resistance (PC1s as described in [7, 8]) to single-shot radiotherapy (RTX), fractionated RTX, TMZ, and TMZ + single-shot RTX are depicted on top by unsupervised hierarchical clustering

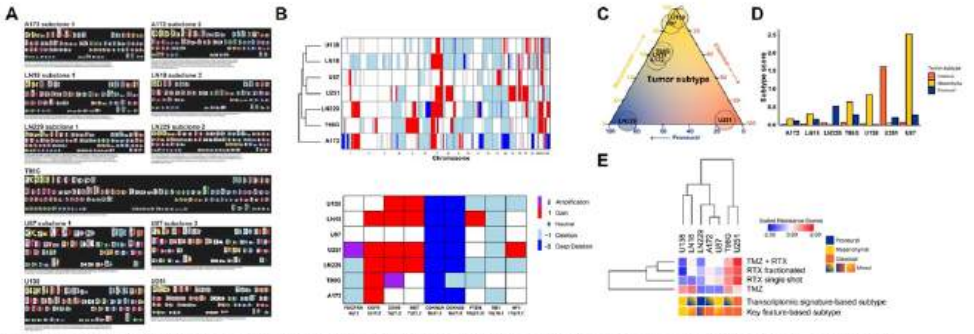

Fig. 2 Molecular subtyping of human glioblastoma cell lines. **a** Spectral karyotyping (SKY FISH) analyses of human glioblastoma cell lines. SKY FISH analyses were performed as previously described [15]. Karyotypes were described according to the international system of human cytogenetic nomenclature (ISCN, edition 2013) [16]. For cell lines A172, LN18, LN229, and U87, two distinct cytogenetic subclones are shown each. **b** Array comparative genomic hybridization (aCGH) analysis of glioblastoma cell lines. Hierarchical clustering of genomic copy number calls of chromosomes 1–22 in glioblastoma cell lines (top), and copy number status of gene loci with known association to glioblastoma subtypes (bottom). Copy number gains (up to 4 copies) are depicted by red bars, copy number amplifications (> 4 copies) by purple bars, and copy number losses by light blue (1 copy), and dark blue (complete loss), respectively. **c** Ternary plot of molecular subtyping of human glioblastoma cell lines on basis of mRNA expression of subtype-related signature genes (according to Wang et al. [23]), obtained by ssGSEA. The proneural subtype is depicted in blue, the classical subtype is depicted in orange, and the mesenchymal subtype is depicted in yellow. Values for subtypes were scaled to sum of 100% per cell line. **d** Bar plots of molecular subtype scores. **e** Graphical presentation of molecular subtypes of human glioblastoma cell lines as revealed by transcriptomic signature-based (top) and key feature-based subtyping (bottom), respectively. Scores of inherent therapy resistance are depicted by unsupervised hierarchical clustering

were summed up before being divided by the total number of features (Fig. 2c). Alternatively, molecular subtyping was performed by the single-sample gene set enrichment analysis (ssGSEA) algorithm provided as an R-package by Wang et al. [23], and using the reduced transcriptomic signatures from Verhaak et al. [24] (Fig. 2d). The negative log10 values of the resulting p-values were used as molecular subtype scores, and according to the maximum value the molecular subtype was assigned.

Integration of therapy resistance with microarray expression data

Integration of therapy resistance data with microarray transcriptome data was performed via different alternative approaches. On single gene level, log2 expression values obtained by microarray analyses were subjected to Pearson's correlation with resistance scores as extracted from clonogenic survival data via PCA (Additional file 1: Table S1 and [7, 8]). Similarly, but with focus on cancer-related genes, only members of the cancer gene consensus (CGC), a library of genes stored in the catalogue of somatic mutations in cancer (COSMIC) [25] were used for correlation analyses. Thirdly, integration on gene set level was performed by ranking genes in accordance to their respective correlation coefficients with therapy resistance scores followed by pre-ranked gene set enrichment analyses (GSEAs) [9]. Leading edge genes were visualized by functional interaction networks in Cytoscape (Cytoscape Consortium, San Diego, CA, USA) [26]. Lastly, integration on gene set level was alternatively performed via an inverse workflow in which the complexity of microarray expression data was first reduced by gene set variation analysis (GSVA) [26] in single samples, followed by Pearson's correlation analyses with therapy resistance scores.

Results

Major differences in global mRNA expression patterns do not match with scores of inherent therapy resistance in a panel of human glioblastoma cell lines

The high degree of therapy resistance, the dismal prognosis, and the persisting lack of prognostic and/or predictive factors in glioblastoma demand for the identification of novel stratification markers for treatment responses on one hand, and potential vulnerabilities for combined modality strategies on the other [6, 27, 28]. Very recently, we published a proof-of-concept screen in which scores of inherent treatment resistance of glioblastoma cells, both to IR and TMZ (Additional file 1: Table S1), were correlated with basal mRNA expression levels of genes related to the DNA damage response (DDR)—a promising target to undermine therapy resistance in glioblastoma [7]. We were able to identify several DDR genes

whose mRNA expression levels showed significant positive correlation with inherent therapy resistance, and pharmacological interference with the function of some of the corresponding gene products using specific inhibitors indeed resulted in sensitization of resistant glioblastoma cells to IR or TMZ treatment, respectively [7].

In the present study, we expanded this workflow to the global transcriptomic level and performed gene expression and DNA methylation microarray analyses using the same cell line panel as before (Fig. 1a, [7]). We first employed the transcriptome/methylome profiles of the cell lines to analyze their relatedness to clinical tumor samples, either from glioblastoma or from lower-grade glioma (LGG) patients as available from TCGA (Fig. 1b, c). All glioblastoma cell lines clustered closer to the glioblastoma patient samples than to the LGG samples, both on the transcriptome (Fig. 1b) and the methylome level (Fig. 1c). However, the cell lines were located at the periphery of the glioblastoma sample cluster, indicating a traceable but limited relatedness which may derive from the absence of non-tumor cells in the cell lines, adaptation processes in cell culture, or other reasons, respectively. We next examined whether the most striking transcriptomic differences across the cell line panel can be linked to inherent therapy resistances of glioblastoma cells. However, sample-to-sample correlation analyses on basis of the 100 most differentially expressed genes resulted in clusters of cell lines without obvious associations to therapy resistance (Fig. 1d). As an example, LN18 and T98G cells, the two most closely related cell lines according to sample-to-sample correlation of their expression profiles revealed strong differences in their respective levels of treatment resistance (Fig. 1d, and [7]) which was most obvious for regimens encompassing IR. Vice versa, cell lines with similar levels of treatment resistance, for instance A172 and U87, showed very far relatedness in sample-to-sample correlation of their gene expression profiles (Fig. 1d). Thus, inherent therapy resistance of glioblastoma cells cannot be directly linked to major differences in global mRNA expression patterns, at least in the cell line panel we analyzed here. Obviously, more systematic approaches integrating large-scale molecular (OMICs) data with functional data are needed [4].

Multi-level cytogenetic and molecular characterization of human glioblastoma cell lines allows their classification into defined molecular subtypes

Several classification systems of glioblastoma on different molecular levels have been described [29]. Emerging consensus is the categorization according to genomic and transcriptomic features into three defined subtypes, termed the classical, the proneural, and the mesenchymal subtype [23, 24]. Model systems of these subtypes have been reported to exhibit marked differences in the response to therapy in vitro and in vivo [30, 31], and the mesenchymal subtype was found to be the most refractory. Intriguingly, strong phenotypic plasticity between subtypes could be observed [30, 31], and the transition from the proneural to the mesenchymal subtype was described to be an important driver of therapeutic failure [30–32]. Clinically, the mesenchymal subtype exhibits the most dismal and the proneural subtype the most benign prognosis, suggested to be driven by subtype-specific signaling pathways including DNA damage repair, cell cycle control, mesenchymal cell movement, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling, PI3K/AKT signaling, JAK/STAT signaling, and WNT pathways [23, 29, 33].

We therefore aimed to classify the glioblastoma cell lines of our panel according to these described subtypes. Firstly, we performed spectral karyotyping (SKY FISH) analyses (Fig. 2a and Additional file 1: Table S2). The karyotypes ranged from a near-diploid chromosomal content in U87 cells up to a near-hexaploid one found in T98G (Fig. 2a and Additional file 1: Table S2). We also noticed that some of the cell lines (A172, LN18, LN229, T98G, and U87) displayed different cytogenetic subclones (Fig. 2a and Additional file 1: Table S2) which is indicative for enhanced chromosomal instability (CIN) in these cell lines [34]. Increased CIN, in turn, is known to affect all major aspects of cancer pathogenicity including tumor progression, metastasis formation, and therapy resistance [35–37], and this also holds true for glioblastoma [38, 39]. Mechanistically, elevated CIN leads to gene copy number alterations (CNAs), which in case of affecting oncogenic driver genes can give rise to the aforementioned subtypes [40, 41]. Distinct CNAs were shown to be associated with different molecular subtypes [24, 42, 43], and we therefore performed array comparative genomic hybridization (aCGH) analyses (Fig. 2b and Additional file 1: Table S3). CNAs with documented association to the classical subtype, including amplification of 7p11.2 (EGFR), and focal deletions of 9p21.3 (CDKN2A) [24], were detected in most of the cell lines (Fig. 2b and Additional file 1: Table S3), whereas CNAs with association to the mesenchymal (loss of 17p11.2, NF1) or the proneural subtype (amplification of 4q12, PDGFRA) were only rarely detected. According to our transcriptomic analyses, driver genes of the mesenchymal subtype, such as TRADD, RELB, TNFRSF1A, and CASP1 [24, 42], were widely expressed. On the contrary, expression of genes related to the proneural subtype including NKX2-2, OLIG2, SOX2, and ERBB3 [24, 44–46] was only detected in one cell line given by LN229

(Additional file 1: Table S3), and genes linked to the classical subtype such as NOTCH3, NES, and SMO [47] were expressed heterogeneously across our panel. In synopsis, subtyping on the basis of cytogenetic and transcriptomic key features classified cell lines A172, T98G, and U251 as classical, and U138 as mesenchymal, but failed to deliver clear-cut classifications for cell lines LN18, LN229, and U87 (Fig. 2e and Additional file 1: Table S3).

Subtyping on the basis of transcriptomic signature genes according to Wang et al. [23] revealed a more heterogeneous pattern of subtypes across our cell line panel (Fig. 2c-e). For A172, LN18, and T98G no clear-cut classifications could be obtained (denoted as "mixed"). These cell lines were located on the proneural-mesenchymal axis (Fig. 2c), presumably reflecting the aforementioned plasticity in proneural-mesenchymal transition. For LN229, U87, U138, and U251, on the contrary, clear-cut classifications could indeed be achieved, classifying LN229 as proneural, U251 as classical, and U87 and U138 as resembling the prognostically challenging mesenchymal subtype (Fig. 2c-e). Reasons for the slight prevalence of this subtype among the panel could be excessive clonal selection and adaptation during initial establishment, and long-term cultivation of these cell lines.

Integrating global gene expression data with clonogenic survival data as a strategy to identify new markers of glioblastoma therapy resistance

So far, our study has revealed that the annotation of defined molecular subtypes to established glioblastoma cell lines is challenging and yields different results depending on the molecular level and the key features employed. Furthermore, our data show that no direct association between the degree of therapy resistance and major transcriptomic differences or molecular subtypes can be drawn. Cell lines assigned to the highly treatment-refractory mesenchymal subtype (U138, U87) showed modest (U87) or even high (U138) sensitivity towards therapy, while the cell line U251 representing the classical subtype showed highest resistance (Fig. 2e). We therefore subjected the therapy resistance data and the transcriptomic profiling data of our glioblastoma cell line panel to correlation analyses with different workflows ([7], and Fig. 3). Firstly, utilizing whole transcriptome data, remaining on the single gene level, and setting a cutoff of $|R| \geq 0.9$ for the correlation of therapy resistance scores and log2 microarray expression values disclosed a limited set of genes whose expression levels were associated with resistance against IR and/or TMZ treatment. Most strikingly, the highest positive correlation for TMZ treatment ($R > 0.99$) was seen for O^6-methylguanine-DNA-methyltransferase (MGMT), not only confirming the literature [48, 49], but also providing a very strong proof-of-concept for the feasibility of our methodological approach.

This strategy allowed us to identify 14 genes that exhibited significant positive correlation with inherent resistance to at least two of the four tested treatments ([7], and Fig. 3a, b), and 8 genes showing negative correlation (Fig. 3b and Additional file 1: Table S4). Three of the positively correlating genes were even linked to resistance towards three of the four treatments, including single-shot IR, fractionated IR, and single-shot IR plus TMZ (Fig. 3a, b). These genes encoded for alpha-1,4-galactosyltransferase (A4GALT), DNA polymerase alpha 1 (POLA1), a replication-associated DNA polymerase for which specific inhibitors are currently developed [50], and adaptor related protein complex 2 subunit beta 1 (AP2B1).

In order to focus on cancer-related genes, we next took advantage of the Cancer Gene Consensus (CGC) gene collection, which encompasses 1133 genes with documented functions in development and progression of cancer [25]. Using this compilation of reduced complexity compared to the whole transcriptome microarray and lowering the cut-off to $|R| \geq 0.7$ yielded a total of 27 cancer-related genes with positive, and 29 genes with negative correlation with resistance scores for at least two types of treatment (Fig. 4a, b). For more detailed analyses, we concentrated on genes, whose expression levels correlated with resistance against three types of treatment, resulting in 22 genes in total, 11 with positive correlation, and 11 with negative correlation (Fig. 4a, b). We then performed a search for drugs targeting the respective gene products with positive correlation with inherent therapy resistance (Fig. 4a and Additional file 1: Table S5). The most interesting target with the strongest expression dynamics across the cell line panel as identified by this approach was the androgen receptor (AR) which has recently been reported to play an important role in prognosis and therapy resistance of glioblastoma [51–57]. Since AR also has roles in other cancer entities, mostly in prostate and in breast cancer [58, 59], multiple inhibitors and antagonists of AR have been developed and trialed [60], yielding successful therapeutic targeting of the AR in these cancer entities [61, 62]. Another candidate for which targeted drugs with clinical approval in other cancer entities are readily available was the mitogen-activated protein kinase kinase 4 (MAP2K4) [63]. Interestingly, in case of glioblastoma a link between AR and MAPK signaling with regard to therapy resistance has recently been described [64]. Finally, our correlation analyses identified STAT5B as a druggable target [65, 66] whose expression was associated with therapy resistance, yet with rather low expression dynamics across the glioblastoma cell line panel.

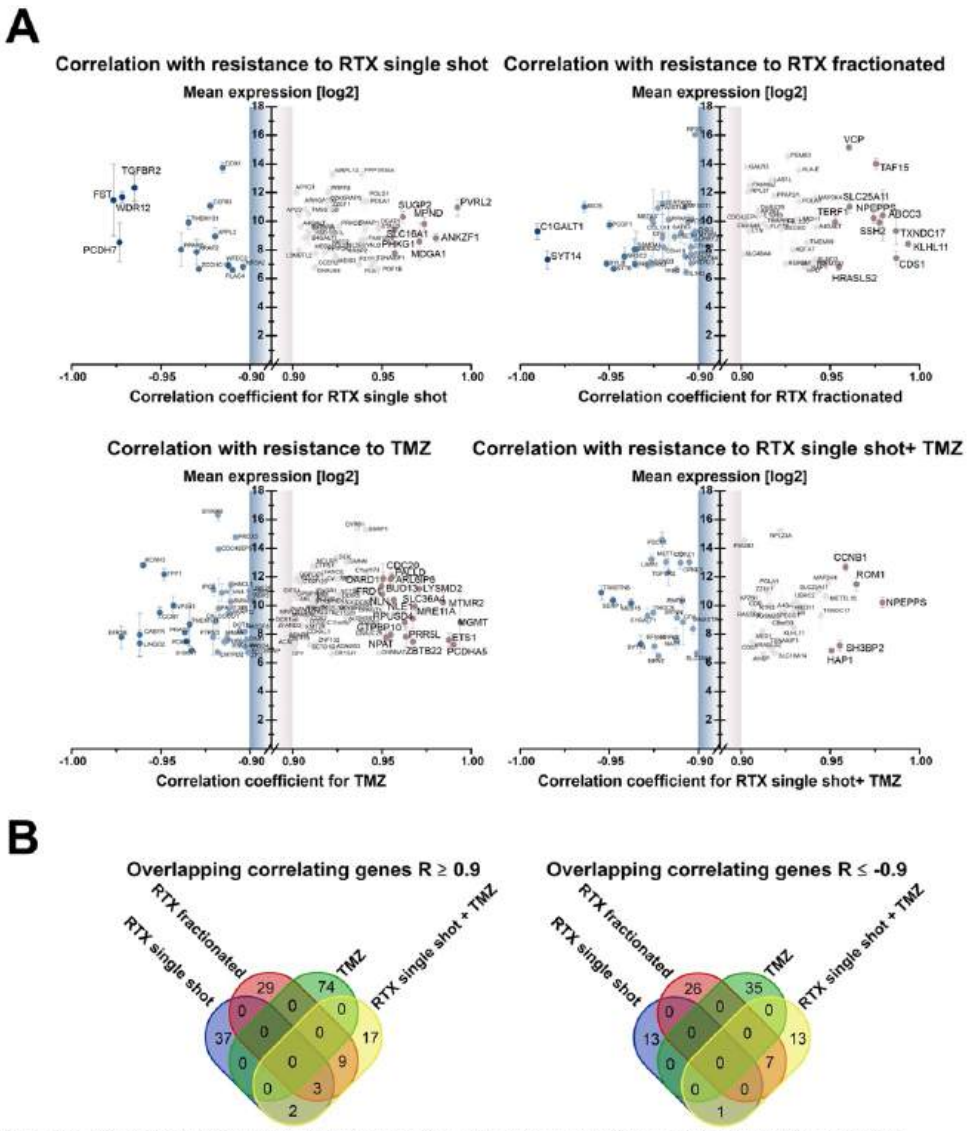

Fig. 3 Integration of global mRNA expression data with scores of inherent therapy resistance in human glioblastoma cell lines. **a** Graphical representation of genes whose log2 mRNA expression levels show strong positive or negative correlation ($|R| \geq 0.9$) with inherent resistance to single-shot RTX, fractionated RTX, TMZ, and single-shot RTX + TMZ in human glioblastoma cell lines. Only genes with an average log2 expression value of 6 or higher compared to astrocytes are shown. **b** Intersect analysis of genes whose log2 mRNA expression shows significant positive or negative correlation ($|R| \geq 0.9$) with inherent resistance to single-shot RTX (blue), fractionated RTX (red), TMZ (green), and single-shot RTX + TMZ (yellow)

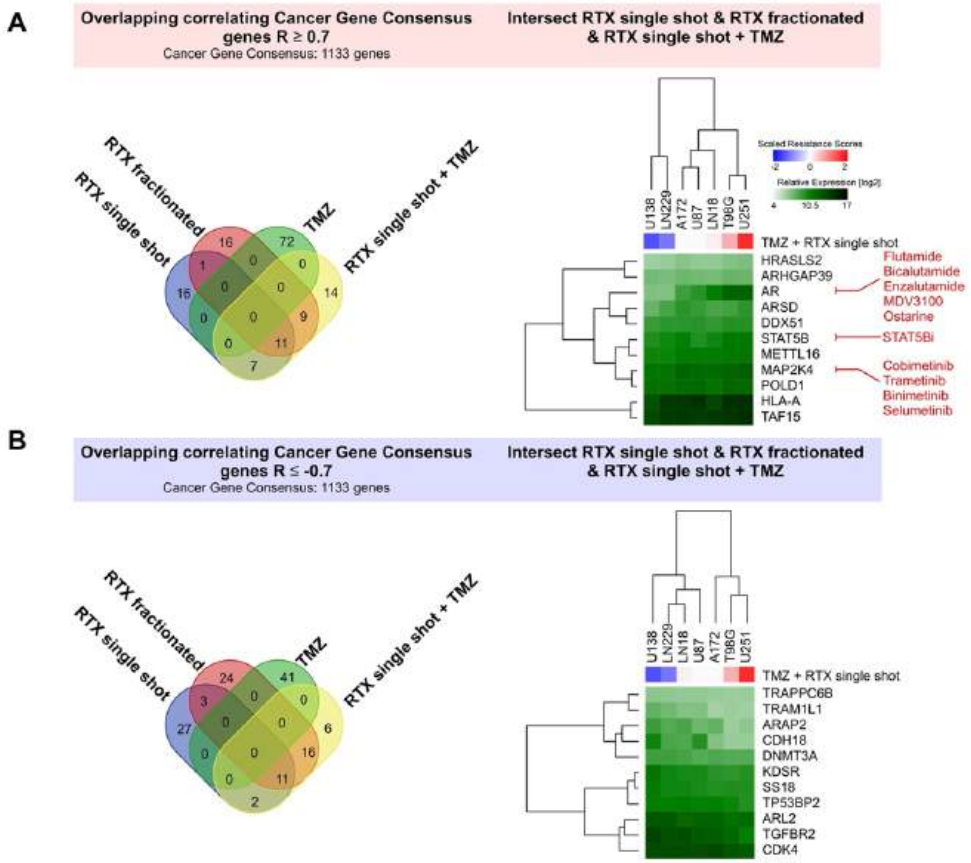

Fig. 4 Integration of mRNA expression data of cancer gene consensus (CGC) genes with inherent therapy resistance in glioblastoma cell lines. **a** Intersect analysis of CGC genes whose log2 mRNA expression levels show significant positive ($R \geq 0.7$) correlation with inherent resistance to single-shot RTX (blue), fractionated RTX (red), TMZ (green), and single-shot RTX + TMZ (yellow, left). Hierarchical clustering of relative log2 mRNA expression levels of 11 intersect genes correlating with resistance to single-shot RTX, fractionated RTX, and single-shot RTX + TMZ (right). Scaled scores of resistance to single-shot RTX + TMZ are depicted, and drugs antagonizing corresponding gene products are indicated in red. **b** Overlap analysis of CGC genes whose log2 mRNA expression shows significant negative ($R \leq -0.7$) correlation with inherent resistance to single-shot RTX, fractionated RTX, TMZ and single-shot RTX + TMZ, and hierarchical clustering of relative log2 mRNA expression levels of 11 intersect genes

Gene set enrichment analyses (GSEAs) identify several signaling circuits as potential contributors to inherent therapy resistance in glioblastoma cells

Stepping from the single gene to the gene set level, we performed pre-ranked gene set enrichment analyses (GSEAs) on the bases of the obtained correlation coefficients (Figs. 3 and 4) and the MSigDB Hallmarks collection which contains ground-truth derived gene sets reflecting the regulation of common biological processes [9]. With an FDR q-value cut-off of ≤ 0.1, a total of 21 gene sets with positive enrichment were found, and 14 gene sets with negative enrichment (Fig. 5a). Gene sets that showed positive/negative enrichment for resistance against at least two of the four treatments included process categories of the core metabolism, such as GLYCOLYSIS and OXIDATIVE_PHOSPHORYLATION, development (ADIPOGENESIS), and immune mechanisms (INTERFERON_ALPHA_RESPONSE), thus

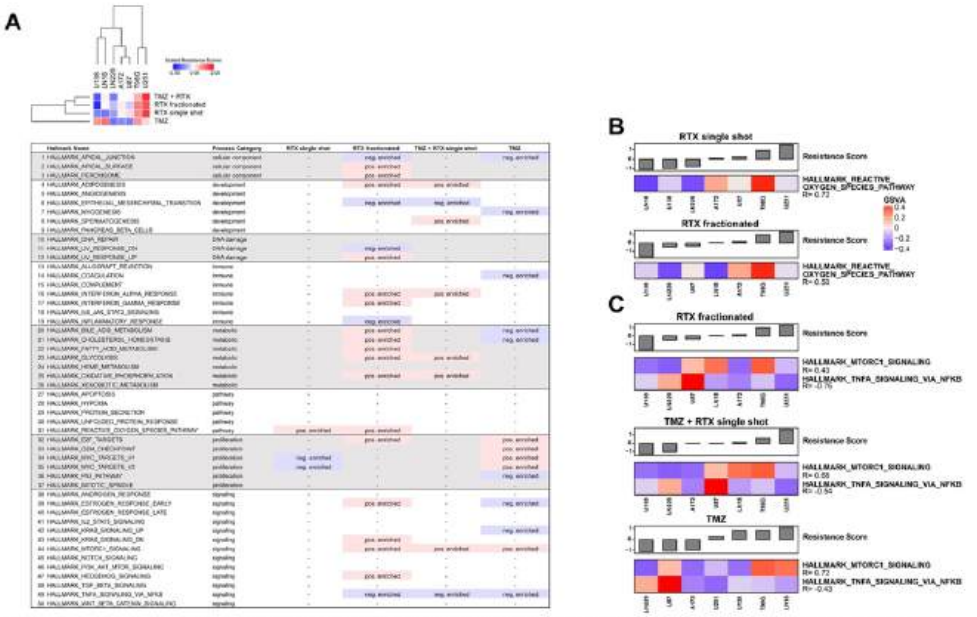

Fig. 5 Gene set enrichment analysis (GSEA) on the basis of genes correlating with therapy resistance identifies pathways of potential contribution to therapy resistance in human glioblastoma cell lines. **a** Tabular presentation of pre-ranked gene set enrichment analysis (GSEA) results on the basis of obtained correlation coefficients (therapy resistance scores vs. gene expression data), and the MSigDB hallmarks collection [9] (FDR q-value cut-off ≤ 0.1). Positively enriched hallmark gene sets are depicted in pink, and negatively enriched hallmark gene sets are depicted in blue. **b, c** Correlation analysis of gene set variation indices as determined by gene set variation analysis (GSVA), and therapy resistance scores to fractionated RTX, single-shot RTX + TMZ, and sole TMZ

according with previous reports [67–73]. Hallmark gene sets found to be negatively enriched with resistance against at least two of the four treatments included EPITHELIAL_MESENCHYMAL_TRANSITION [74, 75] and APICAL_JUNCTION [76] (Fig. 5a). For resistance against treatments comprising IR, the hallmark gene set REACTIVE_OXYGEN_SPECIES_PATHWAY was positively enriched, and the maximal intersect for enrichment with resistance against three treatments was observed for MTORC1_SIGNALING (positive), and TNFA_SIGNALING_VIA_NFKB (negative). Inverting this workflow by first reducing the dimensionality of the expression data by single-sample gene set variation analysis (GSVA), followed by correlation analyses with the obtained GSVA scores and therapy resistance scores basically confirmed these results (Fig. 5b, c).

Leading edge analyses (LEAs) of GSEA-derived gene sets identify druggable candidates and their functional interaction networks

Among the gene sets we identified to be positively or negatively enriched in therapy-resistant glioblastoma cell lines (Fig. 5a, b), we decided to concentrate on REACTIVE_OXYGEN_SPECIES_PATHWAY, MTORC1_SIGNALING and TNFA_SIGNALING_VIA_NFKB [10, 11, 77]. Leading edge analyses (LEAs) were performed by constructing functional interaction networks in Cytoscape. For REACTIVE_OXYGEN_SPECIES_PATHWAY, the leading edge genes comprised a circuit of thioredoxin/peroxiredoxin metabolism and glutathione (GSH) synthesis [78–80] (Fig. 6a and Additional file 1: Table S6) which in view of available drugs also appeared the most interesting vulnerabilities for sensitization in combined modality treatment approaches (Fig. 6b and Additional file 1: Table S6). Corresponding analyses for MTORC1_SIGNALING revealed druggable subnetworks involved in chaperoning, prolyl hydroxylation, proteasomal function, and DNA synthesis and repair, plus

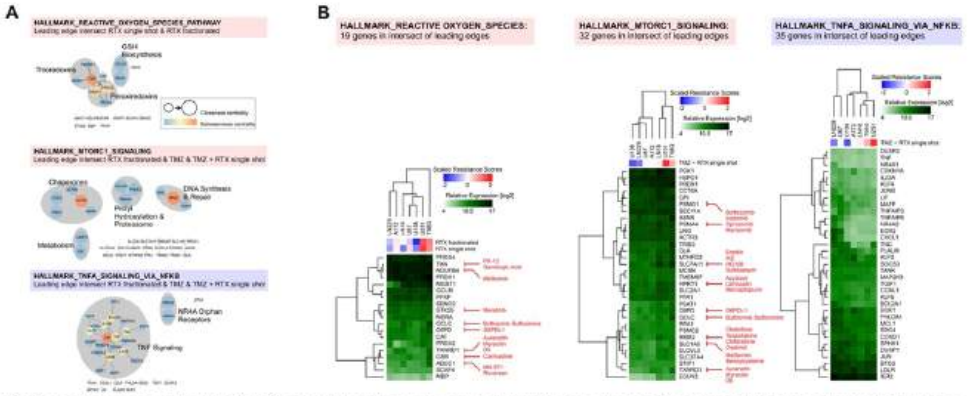

Fig. 6 Leading edge analyses (LEAs) via functional interaction networks and hierarchical clustering. **a** Functional interaction networks of the leading edge intersect genes in hallmark gene sets REACTIVE_OXYGEN_SPECIES_PATHWAY, MTORC1_SIGNALING, and TNFA_SIGNALING_VIA_NFKB as identified by GSEA in Fig. 5A. **b** Hierarchical clustering of relative log2 mRNA expression levels of the respective leading edge intersect genes. Scaled scores of inherent therapy resistances are shown by unsupervised hierarchical clustering, and drugs antagonizing the respective gene products are indicated in red

individual genes overlapping with thioredoxin/peroxiredoxin metabolism and GSH synthesis as disclosed in the leading edge of REACTIVE_OXYGEN_SPECIES_PATHWAY and involved in ferroptosis and autophagy regulation [81, 82] (Fig. 6a, b and Additional file 1: Table S6). Finally, the leading edge of negatively enriched TNFA_SIGNALING_VIA_NFKB was mainly composed of candidates directly involved in TNF/NF-κB signaling [83, 84] (Fig. 6a, b and Additional file 1: Table S6).

Expression of candidate genes for targeted sensitization of glioblastoma as identified by GSEA and LEA is not driven by corresponding CNAs and only marginally anti-correlates with respective promotor methylation

In order to examine if expression of the leading edge genes identified by our correlation/GSEA workflow is reflected by CNAs on the DNA level or by CpG methylation, we integrated the corresponding data sets. Unexpectedly, no significant association between the transcriptome and the CNA level was observed (not

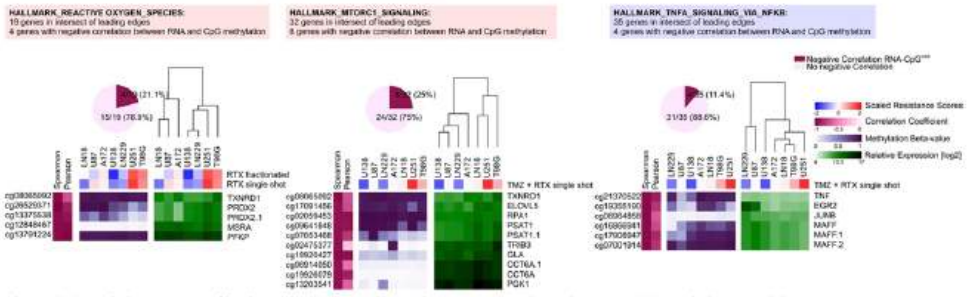

Fig. 7 CpG methylation status of leading edge intersect genes shows poor correlation between DNA methylation and therapy resistance. Correlation analyses of DNA methylation beta values (shown in purple) and relative log2 mRNA expression levels (shown in green) of leading edge intersect genes of the REACTIVE_OXYGEN_SPECIES_PATHWAY, MTORC1_SIGNALING, and TNFA_SIGNALING_VIA_NFKB hallmark gene sets. Coefficients of significant negative correlation are depicted by heat map clustering (shown in dark pink), and scaled resistance scores to single-shot and fractionated RTX (REACTIVE_OXYGEN_SPECIES_PATHWAY), or single-shot RTX + TMZ (MTORC1_SIGNALING, and TNFA_SIGNALING_VIA_NFKB) are shown by unsupervised hierarchical clustering

shown). Furthermore, significant negative correlation with promotor methylation was only observed for a minor subset of genes (Fig. 7) suggesting that other mechanisms dominate gene expression in this context and that integrative approaches such as the one described in the present study require mRNA expression data, rather than CNA or DNA methylation data.

In summary, our data show that workflows correlating mRNA expression data with PCA-derived scores of inherent therapy resistance, followed by GSEA and LEA can reveal therapy resistance markers and potential vulnerabilities for pharmacological sensitization—both previously reported ones and hitherto unknown candidates, and thereby can open new perspectives for mechanism-based, combined modality treatment of glioblastoma and probably also of other cancer entities.

Discussion

Inherent therapy resistance is a major challenge in the treatment of various malignancies. Glioblastoma is particularly well-known for its high degree of treatment resistance, accounting for its dismal prognosis [4]. The identification of key regulators that orchestrate this resistance is therefore inevitable in order to disclose novel perspectives of targeted combined modality therapy and to improve treatment outcome [29]. We recently showed that integrating scores of inherent therapy resistance as extracted from clonogenic survival data with mRNA expression data of the DNA damage response is a suitable approach to identify new candidates for targeted sensitization of glioblastoma [7, 8]. Here, we expanded this workflow to global mRNA expression data and additional molecular levels, including DNA methylome and chromosomal CNAs—all collected under treatment-naive conditions which most closely resemble the clinical situation of tissue sampling in form of tumor biopsies and resections. In accordance with our preceding study, the identification of O^6-methylguanine-DNA-methyltransferase (MGMT) as the best correlating candidate among all genes with positive correlation of mRNA expression with resistance to TMZ provided a proof-of-concept for the feasibility of our approach. Furthermore, on the single gene level, we identified the androgen receptor (AR) as a crucial positive correlator with inherent resistance to three of the four types of treatment—a candidate which is already successfully therapeutically addressed in several cancer entities, such as prostate cancer [58, 61, 85], and thus is eligible to rapid evaluation in glioblastoma [52]. Another gene whose expression level correlated positively with resistance to three types of glioblastoma treatment was mitogen-activated protein kinase kinase 4 (MAP2K4) [63]. Various inhibitors of the MAP kinase family have been developed and several of them, including trametinib and cobimetinib, are readily approved for clinical use in other cancer entities [86]. However, it is known that these compounds commonly do not pass the blood brain barrier (BBB) which for instance undermines efficient treatment of brain metastases originating from MAP kinase-driven melanoma [87, 88]. Therefore, new BBB-passing MAP kinase inhibitors such as E6201 are currently in development [89–92]. Our data suggest that these inhibitors may be interesting candidates for targeted sensitization of glioblastoma to IR and/or TMZ.

In order to identify gene sets, networks, and signaling circuits rather than single genes, we made use of pre-ranked gene set analyses (GSEAs) on the basis of the correlation coefficients (gene expression vs. therapy resistance) followed by leading edge analyses (LEAs). This indeed yielded several candidates whose pharmacological targeting appears interesting in the context of targeted radio- and/or chemosensitization of glioblastoma and—more importantly—for which refined drugs are readily available. Among these were the MSigDB hallmark gene sets REACTIVE_OXYGEN_SPECIES_PATHWAY and MTORC1_SIGNALING, both of which comprise candidates that can be well-targeted by various available drugs [93, 94]. Mechanistically, the identified regulatory circuits have major implications for death and/or survival pathways, such as ferroptosis and autophagy [81, 95], and crucial pro-survival players would represent interesting targets in order to break glioblastoma cell death evasion and resistance against IR [96–98] or TMZ [99], respectively.

When tracing the mRNA expression data back to the chromosomal CNA status, we did not observe relevant associations. Furthermore, CpG methylation status did only in part reflect the obtained mRNA expression data. Accordingly, other mechanisms, including posttranscriptional regulation of mRNA expression by microRNAs (miRNAs) [100], may be involved in glioblastoma gene regulation accounting for the observed therapy resistance-associated mRNA expression patterns, and this would also fit with several recent reports identifying miRNA signatures as outcome prognosticators of glioblastoma [101–104]. It is in accordance with previous reports which found only marginal associations between the methylation status of MGMT promotor and mRNA or protein expression levels in glioblastoma patients [49, 105]. Hence, it is feasible to assume that our integrative approach requires mRNA expression data, rather than DNA methylation or CNA data to obtain robust results. In how far (phospho-)proteomic data would further improve the study outcome remains to be investigated [106].

Similar systematic in vitro analyses of therapy resistance on the basis of clonogenic survival data and multi-level molecular data are rare [107, 108], not only in glioblastoma [109–111]. Most studies of similar purpose integrate molecular data from patient material provided by publicly available databases such as TCGA with clinical data in order to identify genes with association to therapy resistance [22, 112–114]. However, the molecular data obtained from such cohorts are often of high complexity given that biopsied/resected patient material usually comprises complex tumor tissue, including tumor stroma and normal tissue cells rather than only tumor cells [115, 116]. Furthermore, information on treatment courses and clinical endpoints commonly is scarce and/or incomplete, thus further hampering the interpretability of these data. Our results do not only provide proof-of-concept for the feasibility of the chosen integrative in vitro approach. The data sets generated in the present study comprising functional (clonogenic survival data) and multi-level molecular data (mRNA transcriptome, DNA methylome, chromosomal CNA, and SKY FISH) of very commonly used glioblastoma cell lines (in an unperturbed, untreated stage) also represent a valuable toolbox which can be readily interrogated by other researchers in the field of glioblastoma therapy resistance. Nevertheless, since dynamic changes on the analyzed molecular levels (particularly on the transcriptome and on the methylome level) are to be expected in response to therapy and may well be causative for the emergence of acquired therapy resistance [30, 117–119], further research is certainly needed. Finally, the integrative nature of the described workflow can be adapted to other disease models, such as 3D cell culture and organoids [120], since this may impact both, global mRNA expression and therapy resistance [121–123].

Abbreviations

A4GALT	Lactosylceramide 4-alpha-galactosyltransferase
ABC	ATP binding cassette
ABCC1	ATP binding cassette subfamily C member 1
aCGH	Array comparative genomic hybridization
ACTR3	Actin related protein 3
AKT	V-Akt murine thymoma viral oncogene
AKT1	V-Akt murine thymoma viral oncogene 1
ANSI	American National Standards Institute
AP2	Adaptor related protein complex 2
AP2B1	Adaptor related protein complex 2 subunit beta 1
AR	Androgen receptor
ARAP2	ArfGAP with RhoGAP domain, ankyrin repeat and PH Domain 2
ARHGAP39	Rho GTPase activating protein 39
ARL2	ADP ribosylation factor like GTPase 2
ARSD	Arylsulfatase D
ASCT2	Alanine serine cysteine transporter 2
ASNS	Asparagine synthetase
ATCC	American Type Culture Collection
BBB	Blood brain barrier
BCL2A1	BCL2 related protein A1
BCR	BTB-Cul3-Rbx1
BTB	BR–C, Ttk and bab
BTG3	BTG anti-proliferation factor 3
CASP1	Caspase-1
CAT	Catalase
C1GALT1	Core 1 glycoprotein-N-acetyl-galactosamine-3-beta-galactosyl-transferase 1
CCND1	Cyclin D1
CCNL1	Cyclin L1
CCT6A	Chaperonin containing TCP1 subunit 6A
CDH18	Cadherin 18
CDK4	Cyclin dependent kinase 4
CDKN1A	Cyclin dependent kinase inhibitor 1A
CDS1	Phosphatidate cytidylyltransferase 1
CFH	Complement factor H
CGC	Cancer gene consensus
CHI3L1	Chitinase-3-like protein 1
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CNA	Copy number alteration
COSMIC	Catalogue of somatic mutations in cancer
CSF1	Colony stimulating factor 1
CUL3	Cullin 3
CXCL1	C-X-C motif chemokine ligand 1
CXCR4	C-X-C motif chemokine receptor 4
D-MEM	Dulbecco's modified eagle medium
DAPI	4′,6-Diamidino-2-phenylindole
DDR	DNA damage response
DDX51	DEAD-box helicase 51
DNMT3A	DNA methyltransferase 3 alpha
DUSP1	Dual specificity phosphatase 1
DUSP2	Dual specificity phosphatase 2
EGFR	Epidermal growth factor receptor
EGLN3	Egl-9 family hypoxia inducible factor 3
EGFRvIII	Epidermal growth factor receptor variant III
EGR2	Early growth response 2
ELOVL5	Fatty acid elongase 5
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FDR	False recovery rate
FIR	Fractionated IR
FOXP3	Forkhead box protein 3
G6PD	Glucose-6-phosphate dehydrogenase
GCKIII	Germinal centre kinase III
GCLC	Glutamate-cysteine ligase catalytic subunit
GCLM	Glutamate-cysteine ligase modifier subunit
GEO	Gene expression omnibus
GLA	Galactosidase alpha
GPI	Glucose-6-phosphate isomerase
GSC	Glioma stem cell
GSEA	Gene set enrichment analysis
GSH	Glutathione
GSR	Glutathione-disulfide reductase
GSSG	Gluthathione disulfide
GSVA	Gene set variation analysis
HLA-A	Major histocompatibility complex, class I, A
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HRASLS2	HRAS-like suppressor 2
HSPD1	Heat shock protein family D member 1
HAP1	Huntingtin-associated protein 1
HTT	Huntingtin
IDO1	Indoleamine 2,3-dioxygenase 1
IER2	Immediate early response gene 2
IL2	Interleukin 2
IL2RA	Interleukin-2 receptor alpha
IL4	Interleukin 4
IL23A	Interleukin 23 subunit alpha
IR	Ionizing radiation
ISCN	International system for cytogenetic nomenclature
JAK	Janus kinase

JNK	C-Jun N-terminal kinase
JUN	Jun proto-oncogene
JUNB	Jun B proto-oncogene
KDSR	3-Ketodihydrosphingosine reductase
KEAP1	Kelch like ECH associated protein 1
KLF2	Kruppel like factor 2
KLF4	Kruppel like factor 4
KLF6	Kruppel like factor 6
KLHL11	Kelch-like family member 11
KLK2	Kallikrein related peptidase 2
KLK3	Kallikrein related peptidase 3
LDLR	Low density lipoprotein receptor
LEA	Leading edge analysis
LGG	Low-grade glioma
LIF	Interleukin 6 family cytokine
LKB1	Liver kinase B1
MAFF	MAF BZIP transcription factor F
MAP	Mitogen-activated protein kinase
MAP2K3	Mitogen-activated protein kinase kinase 3
MAP2K4	Mitogen-activated protein kinase kinase 4
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MC	Mediator complex
MCL1	Induced myeloid leukemia cell differentiation protein Mcl-1
MCM4	Minichromosome maintenance complex component 4
MCM15	Mediator complex subunit 15
METTL16	Methyltransferase 16
MGMT	O^6-methylguanine-DNA-methyltransferase
MGST1	Microsomal glutathione S-transferase 1
miRNA	MicroRNA
MSRA	Methionine sulfoxide reductase A
MTHFD2	Methylene-tetrahydrofolate dehydrogenase 2
mTORC1	Mammalian target of rapamycin complex 1
MYNN	Myoneurin
NDUFB4	NADH-ubiquinone oxidoreductase subunit B4
NES	Nestin
NF-κB	Nuclear factor κB
NF1	Neurofibromin
NOTCH3	Notch receptor 3
NPEPPS	Puromycin-sensitive aminopeptidase M1
NR4A1	Nuclear receptor subfamily 4 group A member 1
NR4A2	Nuclear receptor subfamily 4 group A member 2
NRF2	NF-2-related factor 2
NSCLC	Non-small cell lung cancer
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PDGFR	Platelet-derived growth factor receptor
PDGFRA	Platelet-derived growth factor receptor alpha
PFKP	Phosphofructokinase, platelet
PGK1	Phosphoglycerate kinase 1
PHLDA1	Pleckstrin homology like domain family A member 1
PI3K	Phosphatidylinositol 3-kinase
PLAAT2	Phospholipase A and acyltransferase 2
PLAUR	Plasminogen activator, urokinase receptor
POL1	RNA polymerase 1
POLA1	DNA polymerase alpha 1, catalytic subunit
POLD1	DNA polymerase delta 1, catalytic subunit
POLR1F	RNA polymerase 1 subunit F
PPA1	Inorganic pyrophosphatase 1
PRDX1	Peroxiredoxin 1
PRDX2	Peroxiredoxin 2
PRDX4	Peroxiredoxin 4
PRL	Prolactin
PSAT1	Phosphoserine aminotransferase 1
PSMA4	Proteasomal 20S subunit alpha 4
PSMB3	Proteasomal subunit beta 3
PSMC2	Proteasome 26S subunit, ATPase 2
PSMG1	Proteasome assembly chaperone 1
PSMG2	Proteasome assembly chaperone 2
PSMG4	Proteasome assembly chaperone 4
RAS	Rat sarcoma
RBX1	Ring box 1
RELB	Transcription factor RelB
RING	Really interesting new gene
RNAseq	RNA sequencing
ROS	Reactive oxygen species
RPA1	Replication protein A1
rRNA	Ribosomal RNA
RRM2	Ribonucleotide reductase regulatory subunit M2
RTX	Radiotherapy
SAP	Stress-activated protein
SBNO2	Strawberry Notch homolog 2
SCAF4	SR-related CTD associated factor 4
SDC4	Syndecan 4
SEC11A	SEC11 homolog A
SGK1	Serum/glucocorticoid regulated kinase 1
SKY FISH	Spectral karyotyping fluorescent in situ hybridization
SLC1A5	Solute carrier family 1 member 5
SLC2A1	Solute carrier family 2 member 1
SLC7A11	Solute carrier family 7 member 11
SLC25A11	Solute carrier family 25 member 11
SLC37A4	Solute carrier family 37 member 4
SMO	Smoothened
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signaling 3
SOD1	Superoxide dismutase 1
SOX	Sry-type HMG box
SOX2	Sry-type HMG box 2
SOX10	Sry-type HMG box 10
SPHK1	Sphingosine kinase 1
SS18	SS18 subunit of BAF chromatin remodeling complex
ssGSEA	Single-sample GSEA
SSIR	Single-shot IR
STAT	Signal transducer and activator of transcription
STAT5B	Signal transducer and activator of transcription 5B
STIP1	Stress induced phosphoprotein 1
STK25	Serine/threonine kinase 25
STR	Short tandem repeat
SYPL1	Synaptophysin-like 1
SYPL2	Synaptophysin-like 2
SYT14	Synaptotagmin-14
TAF15	TATA-box binding protein associated factor 15
TANK	TRAF family member associated NF-κB activator
TGFBR1	Transforming growth factor beta receptor 1
TGFBR2	Transforming growth factor beta receptor 2
TGIF1	TGFβ induced factor homeobox 1
TMEM97	Transmembrane protein 97
TMZ	Temozolomide
TNC	Tenascin C
TNF	Tumor necrosis factor
TNFA	Tumor necrosis factor alpha
TNFAIP3	Tumor necrosis factor alpha induced protein 3
TNFAIP6	Tumor necrosis factor alpha induced protein 6
TNFRSF1A	Tumor necrosis factor receptor super family member 1A
TP53BP2	Tumor protein p53 binding protein 2
TRADD	TNFRSF1A associated via death domain
TRAM1L1	Translocation associated membrane protein 1 like 1
TRAPPC6B	Trafficking protein particle complex, subunit 6B
TRIB3	Tribbles pseudokinase 3
TRX	Thioredoxin
TSNAXIP1	Translin-associated X-interacting protein 1
TWISTNB	TWIST neighbor
TXN	Thioredoxin
TXNDC17	Thioredoxin domain containing protein 17
TXNRD1	Thioredoxin reductase 1
UNG	Uracil DNA glycosylase
WNT	Wingless and Int-1
ZDHHC17	Zinc finger DHHC-type palmitoyltransferase 17
ZZEF1	ZZ-type zinc finger and EF-hand domain-containing protein 1

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13014-023-02241-4.

Additional file 1. Supplementary tables.

Additional file 2. Compilation of all molecular data collected in this study.

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Author contributions
LES, DP, PW, NB, DFF, AEN, MS, TH, JH, MN, CB, HZ, KL, KU, and MO conceived the experiments, analyzed the data, and discussed the data. LES, DP, PW, NB, DFF, AEN, MS, TH, JH, KU and MO performed the experiments. LES, KU, and KL prepared the figures, and performed bioinformatical and statistical analyses. MO, LES, KU, and KL wrote the manuscript. All the authors read and approved the final manuscript.

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Availability of data and materials
The gene expression data and the array CGH data presented in this study are publicly available at Gene Expression Omnibus (GEO) under the super set accession number: GSE119637. For the review process, reviewers can access the data under the link https://www.ncbi.nih.gov/geo/query/acc.cgi?acc=GSE119637 using the token 'sfqnocumrvofrmv'.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
All authors declare that there is no conflict of interest.

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