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Epigenetische und Kinom-spezifische Muster als prognostische Marker beim Glioblastom

Dissertation

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1.1. Einleitung und Fragestellung

Das Glioblastom ist einer der häufigsten bösartigen Tumore, die im zentralen Nervensystem (ZNS) einer adulten Person auftreten können. Die Klassifizierung von Tumoren findet über die Weltgesundheitsorganisation (WHO) in 4 Graden statt [1]. Dabei werden Glioblastome als Grad 4 eingestuft, wodurch die höchste Malignität beschrieben wird. Glioblastome treten überwiegend im Großhirn auf, bevorzugt in den Frontal- und Temporallappen [2]. Hirntumore können aufgrund ihrer Ursprungszellen oder den Ähnlichkeiten zu einzelnen Zelltypen in verschiedene Gruppen unterteilt werden. Dabei zählt das Glioblastom zu den Gliomen, weil ihre Zellen eine Ähnlichkeit zu Gliazellen aufweisen [2].
Glioblastome treten meistens in älteren Patienten auf, die das 60. Lebensjahr überschritten haben. Das durchschnittliche Alter liegt bei 64 Jahren, wobei der Tumor bei Männern häufiger diagnostiziert wird als bei Frauen mit einem Verhältnis von 1,6:1 [3, 4].

Um ein Glioblastom zu erkennen, können sowohl histologische als auch bildgebende Verfahren und molekulare Analysen angewandt werden. Histologisch lässt sich ein Glioblastom meist an mikrovaskulärer Proliferation und Nekrose erkennen [5]. Die molekularen Analysen haben sich durchgesetzt, um den Tumor zu subklassifizieren. Dafür wird standardmäßig die Methylierung der *desoxyribonucleic acid* (DNA) analysiert, und mithilfe eines Classifiers, welcher in Heidelberg am Deutschen Krebsforschungszentrum (DKFZ) entwickelt wurde [6, 7], kann die Tumorprobe den verschiedenen Hirntumorentitäten zugeteilt werden. Das Methylom eignet sich aufgrund der klinisch und biologisch hohen Diversität insbesondere für die detaillierte Charakterisierung von Hirntumoren.

Das Glioblastom wird dabei in vier verschiedene Methylierungs-Subgruppen unterteilt. In Tabelle 1 sind die Charakteristika der drei häufigsten Subgruppen erläutert, welche als mesenchymal (MES), Rezeptor Tyrosinkinase (RTK) I und RTK II bezeichnet werden. Innerhalb dieser Arbeit wird sich auf diese drei Subgruppen beschränkt.

Tabelle 1: ***Charakteristika der drei häufigsten GBM-Subgruppen angelehnt an die WHO-Klassifikation von ZNS-Tumoren 2021*** **[8, 9]**

Subgruppe	Charakteristika	Relative Häufigkeit
RTK I	Platelet-derived growth factor receptor alpha *(PDGFRA)* Amplifikation	20%
RTK II	Epidermal Growth Factor Receptor *(EGFR)* Amplifikation	40-50%
MES	Angereicherte Neurofibromatosis type 1 (*NF1)* Aberration	20-45%

In Bezug auf das Überleben zeigten die GBM-Subgruppen keinen signifikanten Unterschied, obwohl die Biologie dieser Subgruppen hinsichtlich der Expression oder Amplifikationen einiger Gene *(SOX10*, *PDFGRA*, *EGFR*), der mittleren Methylierung, der Methylierung einzelner Dinukleotide, bei denen Cytosin auf Guanin folgt (CpG), und auch des mittleren Alters Unterschiede aufweist [8, 10–15]. Die Subklassifizierung von GBM-Proben dient daher nicht als prognostischer Marker.

Glioblastom-Patienten und -Patientinnen werden mittels einer Kombination aus der bestmöglichsten Operation, Chemotherapie und Bestrahlung behandelt, wobei für die Chemotherapie in der Regel Temozolomid (TMZ) verwendet wird. Trotz dieser Kombination liegt das mediane Überleben bei nur 15 Monaten nach der Diagnose [3], wobei 20% der Patienten und Patientinnen nur maximal 9 Monate und weitere 20% mindestens 2,5 Jahre nach der Diagnose überleben [4]. Diese verhältnismäßig große Zeitspanne kann zum Teil durch prognostische Marker beschrieben werden.
Positive prognostische Marker für ein längeres Überleben nach Therapie sind ein verhältnismäßig junges Alter der Patienten und Patientinnen, ein hohes Resektionsausmaß bei der Operation sowie ein methylierter O6-Methylguanin-DNA Methyltransferase *(MGMT)*-Promotor. Gerade im Zusammenhang mit dem Ansprechen auf die TMZ-basierte Chemotherapie spielt der *MGMT*-Promotor-Methylierungsstatus eine entscheidende Rolle [16]. Jedoch ist die Vorhersage des Ansprechens auf die Therapie noch unzureichend und weitere prognostische Marker sind erforderlich, um den Erfolg der individuellen Behandlung genauer abschätzen zu können. Um neue Marker zu identifizieren, bieten sich epigenetische Analysen oder Analysen des Kinoms, des Proteoms oder der Genexpression anhand von *bulk*-Daten oder Einzelzelldaten an.

1. Synopse und Kinom-Analyse

Kinom-Analysen:

Die Daten der Kinom-Analyse wurden im Vorhinein nicht publiziert.

Proteinkinasen sind größtenteils für die Signalübertragung in Zellen verantwortlich, indem sie eine Phosphorylierung von Proteinen einer Zelle vornehmen können [17]. Spezielle Proteinkinasen gehören den Serin/Threonin-Kinasen (STK) oder Proteintyrosin-Kinasen an, die von bedeutender Wichtigkeit für die meisten zellulären Prozesse, wie dem Zellwachstum und der Regulation des Zellzyklus, sind [18]. Außerdem werden sie in Zusammenhang mit vielen malignen Prozessen gebracht [19, 20]. Alle Kinasen einer Zelle zusammengefasst beschreiben das Kinom.

Die Analyse des Kinoms stellt eine Möglichkeit dar, Biomarker und Mechanismen für ein unterschiedliches Therapieansprechen zu finden und personalisierte Therapien voranzubringen. Die Analyse der Kinaseaktivität auf Omics-Ebene mit Hilfe des funktionellen Kinom-Profilings konnte beispielsweise bei Kopf-Hals-Tumoren angewendet werden, um Kinasen zu identifizieren, deren Aktivität mit der Prognose assoziiert ist. Die sich daraus abgeleiteten therapeutischen Targets könnten sowohl dem Verständnis der Tumorausbreitung als auch der Verbesserung der Therapie dienen [19]. Zudem könnte das funktionelle Kinom-Profiling zukünftig auch in der personalisierten Medizin eingesetzt werden, um bereits vor Therapiebeginn die Effektivität von Kinaseinhibitoren vorherzusagen, um dadurch Patienten und Patientinnen individualisiert und effektiv zu behandeln.

Das Ziel dieser Studie war es, Marker in Form von epigenetischen oder Kinom-spezifischen Mustern zu finden, um das Therapieansprechen von GBM-Patienten vorherzusagen. Die anspruchsvolle und intensive Therapie ermöglicht keine Heilung des Tumors und belastet zudem die GBM-Patienten und –Patientinnen, indem sie ihre Aktivität und Selbstbestimmung (Karnofsky-Index) verringern [21]. Dadurch kann es zu einer dauerhaften Verschlechterung der neurologischen Funktionen und der Lebensqualität führen. Es fehlt an ausreichenden Informationen, die das Tumorwachstum und das Therapieansprechen beschreiben, um zukünftig die bestehende Therapie effizient und personalisiert einzusetzen und neue Therapien zu entwickeln. Um zusätzliche Informationen zu generieren und Biomarker für das Ansprechen zu identifizieren, sollten innerhalb dieses Projektes sowohl Kinom- als auch Methylom-Analysen von Tumorproben und GBM-Zelllinien durchgeführt werden.

1.2. Material und Methodik

1.2.1 Kits und Arrays

Tabelle 2: Liste der verwendeten Kits und Arrays

Item	Hersteller
Nucleo Spin® Tissue Kit	Macherey-Nagel, Germany
PamChips	PamGene
Methylation array (450k und EPIC)	Illumina, USA

1.2.2 Software

Tabelle 3: Liste der verwendeten Software

Software	Version	Manufacturer/Source
Graph Pad Prism	6.07	GraphPad Software Inc, USA
R	3.3.3/4.1.1/4.1.2	Open Source, www.r-project.org
RStudio Desktop	2023.06.2 "Mountain Hydrangea"	Posit Software, USA
BioNavigator		PamGene

Für die Analyse wurden verschiedene bioinformatische und statistische Auswertungs-Pakete in R verwendet, die in Tabelle 4*: Für die Analyse verwendete Pakete in RAbbildung 4* näher beschrieben sind.

Tabelle 4: Für die Analyse verwendete Pakete in R

Paket	Version	Funktion
ChAMP	2.30.0	Suche nach signifikanter CpG-Dinukleotide
ComplexHeatmap	2.16.0	Erstellung von Heatmaps
EpiDISH	2.16.0	Dekonvolutionsanalyse von Methylomdaten zur Anteilsbestimmung von Zellpopulationen
ggplot2	3.4.3	Datenvisualisierung
ggpubr	0.6.0	Datenvisualisierung
limma	3.40.0	Batch-Korrektur
maxstat	0.7-25	Multivariate Analyse
minfi	3.9	Preprozessierung der Methylom-Daten
sesame	1.18.4	Preprozessierung der Methylom-Daten
survival	3.5-7	Analyse von Überlebensdaten
survminer	0.4.9	Erstellung von Kaplan-Meier-Kurven

1.2.3 Zelllinien

Die Charakteristika der Zelllinien (ZL) werden in Tabelle 5 beschrieben. Insbesondere das Wachstumsverhalten der Zelllinien unterschied sich zwischen den beiden verwendeten Kohorten und Laborорten. Bis auf zwei Zelllinien wuchsen in Tübingen (Stammzell-ähnliche Zelllinien) alle Zelllinien in Suspension. In Hamburg (etablierte Zelllinien) wuchsen alle Zelllinien adhärent. Es konnten nicht alle Zelllinien für alle Analysen verwendet werden.

Tabelle 5: Charakteristika der Zelllinien-Kohorten

Zelllinie	Laborort	Kohorte	Analyse	Wachstumsverhalten
AR6	Tübingen	Stammzellen	Methylom	Suspension
AR8	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK3	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK7	Tübingen	Stammzellen	Kinom + Methylom	Adhärent
LK11	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK12	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK13	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK17	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK19	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK21	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK23	Tübingen	Stammzellen	Kinom + Methylom	Adhärent
LK28	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK31	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK32	Tübingen	Stammzellen	Kinom + Methylom	Suspension
LK39	Tübingen	Stammzellen	Methylom	Suspension
BS153vIII-	Hamburg	Etablierte ZL	Kinom + Methylom	Adhärent
CAS-1	Hamburg	Etablierte ZL	Kinom + Methylom	Adhärent
DKMGvIII-	Hamburg	Etablierte ZL	Kinom + Methylom	Adhärent
LN71	Hamburg	Etablierte ZL	Kinom + Methylom	Adhärent
LN229	Hamburg	Etablierte ZL	Kinom + Methylom	Adhärent
LN827	Hamburg	Etablierte ZL	Kinom + Methylom	Adhärent
U343	Hamburg	Etablierte ZL	Kinom + Methylom	Adhärent
U87MG	Hamburg	Etablierte ZL	Kinom + Methylom	Adhärent

1.2.7 Methylom-Analyse

Für die Analyse des Methyloms wurde DNA aus Proben (Zelllinien, Tumoren) extrahiert und anschließend das andernorts beschriebene Protokoll verwendet [6]. Für die Analyse des Methyloms wurde der Illumina 450k (450.000 CpG-Dinukleotide) oder EPIC (850.000 CpG-Dinukleotide) array verwendet. Die Prozessierung der daraus entstehenden IDAT-Dateien erfolgte mit dem minfi Bioconductor Paket in R.

1.2.8 Kinom-Analyse

Für die Analyse des Kinoms werden zunächst Proteinlysate benötigt, welche mit dem PamGene-Kit erstellt werden. Anschließend werden die Lysate aufgereinigt und auf die PamChips gegeben, welche jeweils vier Arrays besitzen. In dieser Studie wurden ausschließlich PTK-PamChips verwendet. Drei PamChips können pro Durchgang verwendet werden. Um einen technischen Fehler auszuschließen, läuft in allen Durchgängen die gleiche Kontrollprobe mit. Zusätzlich werden in einem Durchgang pro PamChip die gleichen drei Test-Proben eingespeist, sodass auf jedem Chip eine Kontrolle und drei Test-Proben liegen. Jeder PTK-Array besitzt 196 Peptidsequenzen, welche Phosphorylierungsstellen beinhalten, die mit einer oder mehr Kinasen assoziiert werden. Die Kinase-Aktivität kann anhand der Phosphorylierung und daraus resultierender Fluoreszenz gemessen und analysiert werden. Anschließend erfolgt die bioinformatische Datenanalyse mit einem Tool von PamGene (BioNavigator). Der Aufbau wird in Abbildung 1 dargestellt [22].

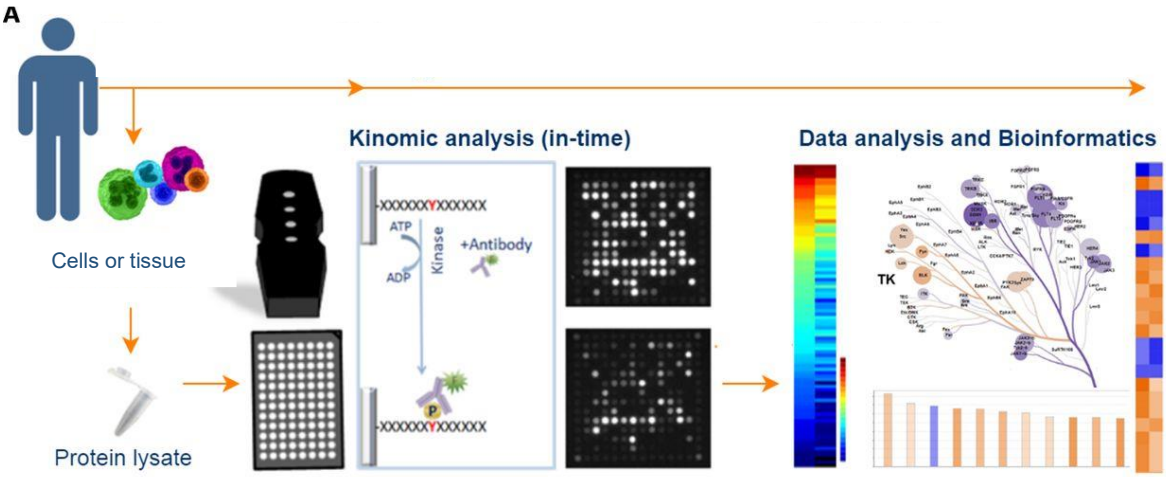

Abbildung 1: Kinom-Analyse: Vom Protein-Lysat zur bioinformatischen Auswertung (adaptiert von [22]). *Das Lysat wurde entweder aus Tumorgewebe oder Zelllinien gewonnen.*

1. Synopse und Kinom-Analyse

In der bioinformatischen Auswertung wurden die Kinase-Aktivitäten auf die Kontrolle des entsprechenden Durchgangs normalisiert, um innerhalb der verschiedenen Durchläufe keinen *bias* zu erhalten.

1.3. Ergebnisse

1.3.1 Methylom-Analyse

Eine Korrelationsanalyse von klinischen Parametern mit Methylomdaten wurde gemacht, um neue Erkenntnisse bezüglich des Therapieansprechens zu bekommen. Dafür wurde das Resektionsausmaß von 305 Patienten und Patientinnen, welche sowohl Chemotherapie als auch Bestrahlung erhalten haben, mit den Überlebensdaten korreliert, wobei das Ausmaß in *gross total resection* (GTR), *near* GTR und partiale Resektion (PR)/Biopsie unterteilt wurde. Patienten und Patientinnen, die eine Resektion von mindestens 90% des kontrastverstärkenden Tumorteils erhielten, überlebten signifikant länger als diejenigen mit einem geringeren Resektionsausmaß (Publikation 2 [15]). Bei Betrachtung der GBM-Subgruppen konnte man diese signifikante Korrelation nur für RTK I und RTK II erkennen, während die MES-Subgruppe keinen signifikanten Unterschied im OS zeigte.
Um einen Zusammenhang zwischen den Subgruppen und dem Resektionsausmaß weiter zu untersuchen, wurden 68 Tumorproben von Patienten und Patientinnen, bei denen ein lokales Rezidiv auftrat und eine zweite Tumorresektion durchgeführt wurde, mit Tumorproben ohne zweite Resektion verglichen. Sowohl für alle Patienten und Patientinnen als auch in den Subgruppen RTK I und RTK II waren signifikante Unterschiede ersichtlich, indem eine wiederholte Resektion mit einem signifikant besseren Überleben korrelierte. Nur für die MES-Subgruppe ergab sich, dass eine zweite Resektion keinen signifikanten Vorteil für das Überleben brachte (Publikation 2 [15]).

Zusätzlich wurden für die Suche nach therapierelevanten Clustern, die das Ansprechen auf die Therapie vorhersagen sollen, die Methylomdaten von 492 Patienten und Patientinnen verwendet, die sowohl Chemotherapie als auch Bestrahlung erhalten haben (Publikation 1 [14]). Diese Patientenkohorte beinhaltete teilweise Patienten und Patientinnen, die in der beschriebenen Resektionsanalyse eingeschlossen waren. Anschließend wurde die Kohorte in länger und kürzer überlebende Patienten und Patientinnen eingeteilt, wofür verschiedene Schwellenwerte verwendet wurden. Bei allen Analysen konnten keinerlei signifikante Muster identifiziert werden, die auf ein Therapieansprechen hinweisen würden (Publikation 1 [14]). Ebenso konnten in GBM-Zelllinien, deren Ansprechen auf Bestrahlung bestimmt wurde, keine signifikanten Muster entdeckt werden.

Dahingegen stellte sich die mittlere globale Methylierung als unabhängiger prognostischer Marker heraus, wobei eine höhere mittlere Methylierung mit einem signifikant besseren Überleben korreliert [14]. In den Subgruppen RTK I und RTK II konnten signifikante Unterschiede im Überleben der Patienten und Patientinnen, welche anhand der mittleren

Methylierung stratifiziert wurden, bestimmt werden, während die MES-Subgruppe keinen signifikanten Unterschied aufwies.
Die mesenchymale Subgruppe zeigte demnach weder einen signifikanten Überlebensvorteil auf Grund eines hohen Resektionsausmaßes, einer wiederholten Resektion bei einem lokalen Rezidiv oder einer erhöhten mittleren globalen Methylierung.

Inwiefern die mittlere globale Methylierung mit einem Ansprechen auf Strahlentherapie korreliert, wurde mithilfe von 23 Zelllinien und einer weiteren Patienten-Kohorte, die keine Chemotherapie und nach der Tumorresektion oder –biopsie ausschließlich Strahlentherapie erhielten, analysiert. Strahlenresistente Zelllinien zeigten dabei eine niedrigere mittlere Methylierung als sensitive Zelllinien. Zudem zeigten auch die Patienten und Patientinnen mit einer höheren Methylierung ein signifikant längeres Überleben, was auf ein besseres Ansprechen auf die Strahlentherapie hindeutete. In Kombination mit den bereits bekannten Markern war die mittlere Methylierung ein zusätzlicher unabhängiger Biomarker für die Vorhersage des Therapieansprechens von Glioblastomen und somit für das Überleben der Patienten und Patientinnen [14].

Zusätzlich zur Analyse des Therapieansprechens sollte geklärt werden, ob rekursive GBM-Tumore eine unterschiedliche mittlere Methylierung als der jeweilige Primärtumor aufwiesen. Schließlich ist bekannt, dass rekursive GBM einen aggressiveren Phänotyp zeigen [23]. Dafür wurden 31 Tumorpaare aus Erst- und Zweitresektionen verwendet und anhand ihrer mittleren globalen Methylierung untereinander verglichen. Es konnte gezeigt werden, dass die Proben einer zweiten Resektion eine signifikant niedrigere Methylierung als die der ersten Resektion aufwiesen. Dieses Ergebnis ist unabhängig von einem Subgruppenwechsel zur RTK I-Subgruppe, die bekannterweise eine niedrigere Methylierung als die anderen beiden Subgruppen aufwiesen [13].

1.3.2 Kinom-Analyse

Die Daten der Kinom-Analyse wurden im Vorhinein nicht publiziert.
Für die Kinom-Analyse wurden die Überlebensdaten nach Bestrahlung der Zelllinien verwendet, die bereits für die Methylom-Analyse genutzt wurden. Diese Zelllinien wurden an verschiedenen Standorten auf deren Strahlensensitivität getestet (Hamburg (n=8) und Tübingen (n=15) [24]) und anschließend als eine Kohorte zusammengefasst. Die Zelllinien wurden in strahlensensitiv und -resistent unterteilt, um anschließend das Kinom der jeweiligen Gruppen miteinander zu vergleichen. Insgesamt konnten 21 der 23 Zelllinien analysiert werden, die bereits für die Methylom-Analyse verwendet wurden (Publikation 1 [14]). Die Cluster-Analyse der Peptid-Phosphorylierung in Relation zur jeweiligen Kontrollprobe

(Abbildung 2) zeigte, dass sich die Zelllinien in 3 Hauptcluster unterteilen lassen. Dabei wird Cluster C durch zwei resistente Zelllinien beschrieben (LK7, LK23), die sich von allen anderen Zelllinien abgrenzten. Diese beiden Zelllinien beschrieben die einzigen adhärent wachsenden Zelllinien der Stammzell-Kohorte, wodurch ein Batch-Effekt in dieser Gesamtkohorte nicht auszuschließen ist. Die Untersuchung eines biologischen Effekts ist für diese beiden Zelllinien nicht möglich. Die anderen beiden Hauptcluster wurden durch 9 und 10 Zelllinien beschrieben, welche zu mindestens 78% aus nur resistenten (7/9 resistente Zelllinien) oder nur sensitiven Zelllinien (8/10 sensitive Zelllinien) bestanden. Die Cluster A und B ließen sich wiederum klar in die jeweiligen Kohorten bzw. das Wachstumsverhalten unterteilen.

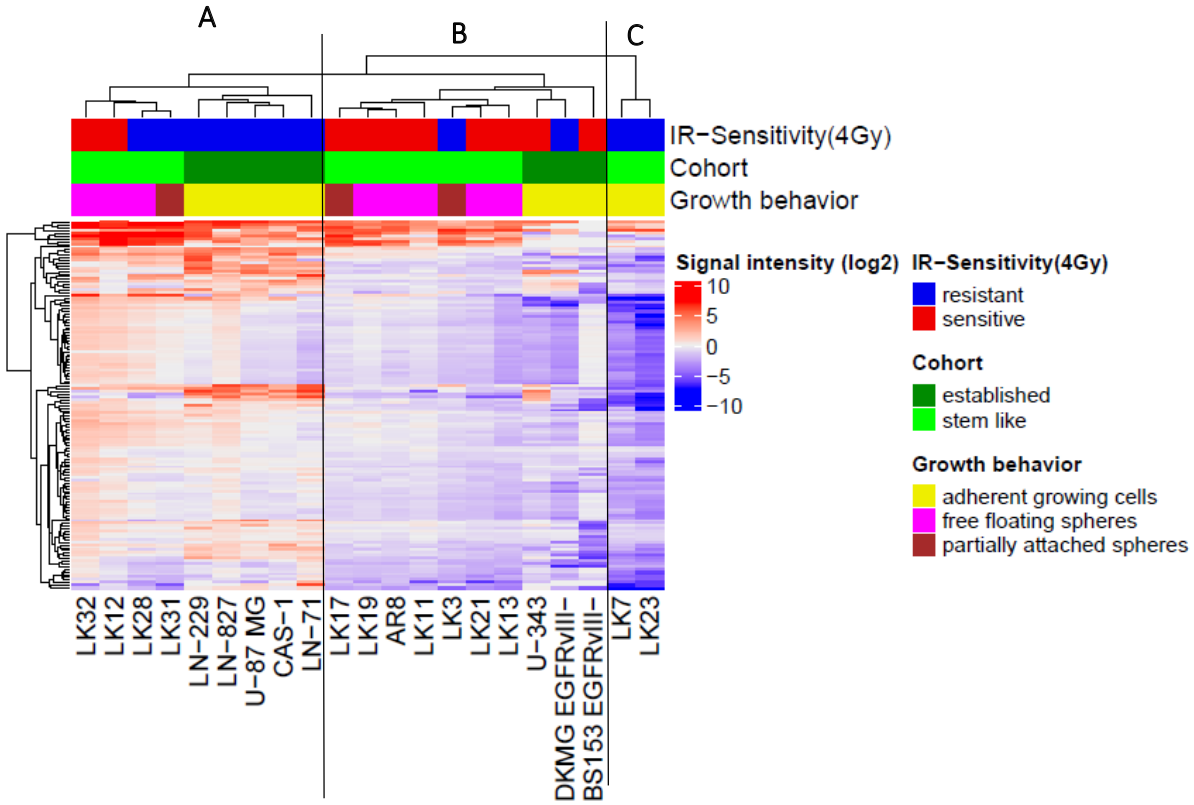

Abbildung 2: Hierarchisches Cluster der relativen Phosphorylierung von 21 GBM-Zelllinien. *Die einzelnen Zelllinien werden in den Spalten dargestellt, während die Peptide in den Zeilen gezeigt werden. Die Annotation der Zelllinien (Strahlensensitivität, Kohorten, Wachstumsverhalten) wird in den obersten drei Zeilen dargestellt. Die Heatmap teilt sich in drei Hauptcluster (A, B, C) auf.*

Bei Betrachtung der mittleren relativen Peptid-Phosphorylierung in Abhängigkeit von der Strahlensensitivität konnte lediglich eine Tendenz, dass resistente GBM-Zelllinien mit einer erhöhten Peptid-Phosphorylierung einhergeht, identifiziert werden. Ein signifikanter

Unterschied zwischen sensitiven und resistenten Zelllinien konnte nicht bestimmt werden. (Abbildung 3). Hierfür wurden die Zelllinien LK7 und LK23, die sich vermutlich aufgrund der Kohorte und des Wachstumsverhalten zu unterschiedlich verhalten, bereits aus der Analyse entfernt.

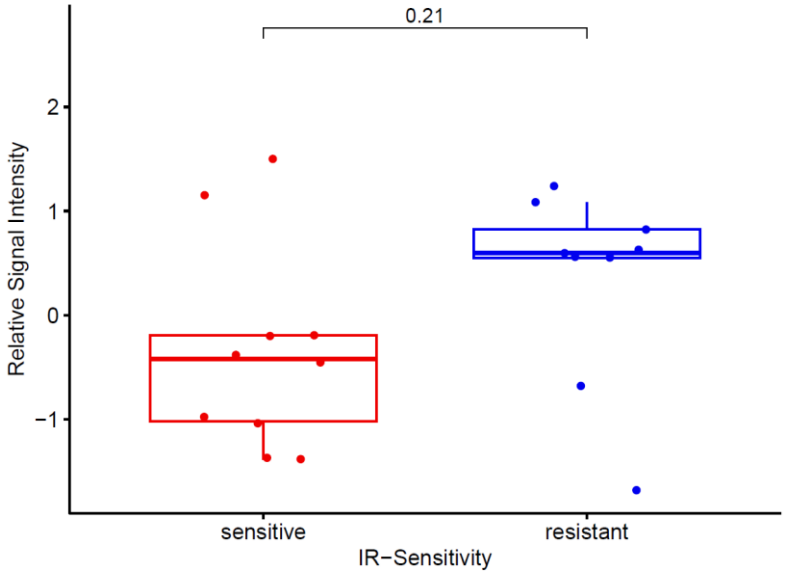

Abbildung 3: Mittlere relative Kinase-Aktivität von 19 Zelllinien, die anhand ihrer Strahlensensitivität unterteilt wurden.

Im Falle der Stammzell-ähnlichen Zelllinien (Tübinger-Kohorte) konnten auch die korrespondierenden Tumore analysiert werden, aus denen die Zelllinien generiert wurden. Der Vergleich der Zelllinien zu den Tumoren mittels einer hierarchischen Cluster-Analyse zeigte, dass die Tumore eine größere Ähnlichkeit untereinander zeigten als zu den entsprechenden Zelllinien (Abbildung 4). Dabei konnten zwei Hauptcluster identifiziert werden, wobei Cluster 1 eine höhere Phosphorylierung der einzelnen Peptide zeigte. Beide Hauptcluster ließen sich in jeweils zwei weitere Subcluster unterteilen (A und B). Das Cluster 1 trennte sich genau nach der Art der Probe auf, indem 1A ausschließlich aus 10 Tumoren und 1B ausschließlich aus 4 Zelllinien bestand. Das zweite Cluster beinhaltete bis auf eine Tumorprobe ausschließlich Zelllinien. Cluster 2B umfasste nur die beiden Zelllinien LK7 und LK23, welches bereits in vorherigen Analysen aufgefallen war und vermutlich auf einen Batch-Effekt bezüglich des Wachstumsverhaltens zurückzuführen war.

Schließlich waren die Unterschiede der Tumoren- und Zelllinien-Kohorten zu groß, weshalb ein Vergleich zwischen Ursprungstumor und der dazugehörigen Zelllinie nicht möglich war, um Ergebnisse der Zelllinien auf den Tumor zu übertragen.

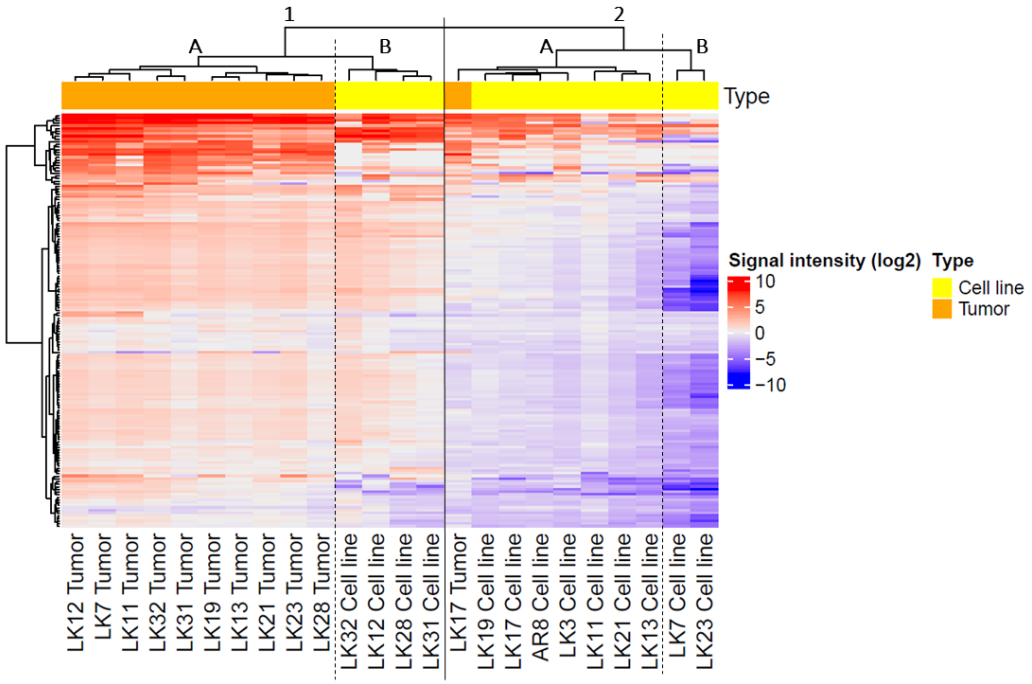

Abbildung 4: Cluster-Analyse der Zelllinien und den dazugehörigen Tumoren. *Die obersten drei Zeilen beschreiben den Probennamen, die Kohorte (Zelllinie oder Tumor) und die Strahlensensitivität. Die Phosporylierung der einzelnen Peptide wird in der Heatmap dargestellt.*

1.4. Diskussion

Die GBM-Patienten und -Patientinnen haben im Vergleich zu anderen Tumor-Patienten und -Patientinnen eine bedeutend schlechtere Überlebenswahrscheinlichkeit und können in aller Regel nicht geheilt werden. Das mediane Überleben liegt bei 15 Monaten, wenn eine Kombination aus der bestmöglichen Resektion des Tumors, Strahlentherapie und Chemotherapie als Therapie angewandt wird. Diese Studie sollte dem Zweck dienen, Marker in Form von epigenetischen oder Kinom-spezifischen Mustern zu finden, damit das Therapieansprechen von GBM-Patienten und -Patientinnen besser vorhergesagt werden kann.

1.4.1 Methylom-Analyse

Als robuste Methode wird die DNA-Methylom-Analyse bereits in der Diagnostik verwendet, und mit ihrer Hilfe konnten schon diverse Biomarker identifiziert werden [6, 7]. Innerhalb dieser Studie konnte zusätzlich die mittlere globale Methylierung als unabhängiger prognostischer Marker identifiziert werden. Sowohl bei Patienten und Patientinnen, die eine Kombinationstherapie aus Bestrahlung und Chemotherapie erhalten haben, als auch welche, die nur Bestrahlung erhielten, konnte ein signifikanter Unterschied im Überleben beobachtet werden (Publikation 1 [14]). Die Zelllinien-Daten konnten diese Erkenntnisse ebenfalls unterstützen: Strahlensensitive Zelllinien zeigten eine höhere mittlere globale Methylierung als strahlenresistente Zelllinien. Es bedarf weiterführender Arbeiten, um die biologischen Grundlagen für diese Beobachtung zu entschlüsseln und eine Validierungskohorte von Patienten und -Patientinnen, die nur Chemotherapie erhalten haben, zu nutzen. Dadurch könnte eventuell gezeigt werden, dass die Beobachtung in der Gesamtkohorte ausschließlich auf die Strahlentherapie zurückzuführen ist.

Zusätzlich zu bereits bekannten Biomarkern ist bekannt, dass sich die GBM-Tumore in hauptsächlich drei Subgruppen teilen lassen, die untereinander verglichen keinen signifikanten Unterschied im Überleben zeigen [12, 13]. Zudem scheinen sich die GBM-Subgruppen in Histologie und der Aggressivität den RTK I- und RTK II-Glioblastomen sehr zu ähneln. Dennoch zeigte die MES-Subgruppe als einzige Subgruppe keinen Unterschied sowohl bezüglich des Resektionsausmaßes als auch bezüglich der mittleren globalen Methylierung auf (Publikation 1 [14] und Publikation 2 [15]). Außerdem ist bekannt, dass ein mesenchymaler Subgruppenwechsel vom Primärtumor zum Rezidiv mit einem schlechteren Therapieansprechen zusammenhängt [25, 26]. Dass sich die MES-Subgruppe nicht anhand ihrer mittleren Methylierung in lang und kurz überlebende Patienten einteilen lässt, könnte mit einer Strahlenresistenz dieser Subgruppe zusammenhängen [26]. Trotz Ähnlichkeiten zwischen den Subgruppen gibt es diverse Unterschiede, die auch von hoher klinischer und

therapeutischer Relevanz sind. Damit könnten GBM-Patienten und -Patientinnen dieser Subgruppe unnötige Therapien erspart bleiben, die eventuell nicht lebensverlängernd sind, aber die Lebensqualität der Patienten und Patientinnen beeinträchtigt [21].

Inwiefern die mittlere Methylierung ein gleich hohes Maß im gesamten Tumor ist, konnte nicht geklärt werden. Denn die mittlere Methylierung wird von einem kleinen Tumorstück bestimmt, welches entnommen wird und welchen Einfluss die intratumorale Heterogenität hat, das heißt, ob der im Gehirn verbleibende Tumoranteil dieselben Charakteristika wie das analysierte Material aufzeigt, bleibt zu untersuchen [27]. Hierfür wären Analysen aus verschiedenen Bereichen von Tumoren sinnvoll, um diese anlässlich ihrer mittleren globalen Methylierung zu analysieren und mit Unterschieden im Therapieansprechen zu korrelieren. Zusätzlich konnte gezeigt werden, dass sich die mittlere Methylierung zwischen Erst- und Zweitresektion signifikant unterschied (Publikation 1 [14]), was auf einen aggressiveren Phänotyp hinweisen könnte. Dennoch ist der zugrunde liegende Wachstumsmechanismus vom Primärtumor zum Rezidiv nicht geklärt, weshalb die Frage, ob ein Tumor bereits resistente Zellen umfasst, die nach der Therapie wachsen können, oder ob einzelne Zellen während der Therapie resistent werden, weiterhin unbeantwortet bleibt [28, 29]. Die Analyse der mittleren Methylierung in verschiedenen Bereichen des Tumors mit der Histologie zu verbinden und gegebenenfalls (Einzelzell-)RNA- oder Einzelzell-Methylom-Analysen zu ergänzen, würde die Forschung dahingehend voranbringen, ob ein aggressiverer Klon mit einer niedrigen mittleren Methylierung für das Rezidiv verantwortlich ist. Zudem könnten histologische und genexpressionistische Charakteristika des Tumors analysiert werden.

1.4.2 Kinom-Analyse

Die Methode der Kinom-Analyse ist weniger robust als die Methylom-Analyse, wird dennoch in bisher wenigen Fällen für Inhibitions-Experimente für einzelne Patienten und Patientinnen genutzt [30]. Für diese Studie wurden zwei Kohorten analysiert, die überwiegend aus Zelllinien bestanden, um nach allgemeinen Biomarkern zu suchen, indem die Strahlensensitivität untersucht wurde. Eine Tendenz, dass strahlenresistente Zelllinien eine höhere Kinase-Aktivität besitzen, konnte ermittelt werden. Dennoch war die Korrelation zwischen der Strahlensensitivität und der Peptid-Phosphorylierung nicht signifikant, was auf die empfindliche Kinom-Analyse zurückzuführen war. Bereits Unterschiede im Wachstumsverhalten, in der Handhabung in unterschiedlichen Laboren oder in der Art der Zelllinien (etabliert oder Stammzell-ähnlich) könnten die Kinase-Aktivität beeinflussen. Zudem traten zu große Unterschiede zwischen Zelllinien und den entsprechenden Ursprungstumoren auf, sodass eine Analyse des Therapieansprechens eventuell auf den eigentlichen Tumor nicht anzuwenden wäre. Notwendig sind Kohorten, die sehr ähnliche Herkunfts-, Wachstums-

und Laborbedingungen haben und dennoch groß genug sind, um statistische Auswertungen zwischen resistenten und sensitiven Zelllinien oder Tumoren zu machen.

1.4.3 Outlook

Die Korrelation der mittleren globalen Methylierung mit der Strahlenempfindlichkeit sollte *in-vitro* weiter getestet werden, indem das Therapieansprechen der Zelllinien nach Behandlung mit einem Medikament, das die mittlere Methylierung erhöht oder senkt, analysiert wird. Dadurch könnte experimentell die Korrelation der mittleren DNA-Methylierung zur Strahlensensitivität auf eine Kausalität hin untersucht werden. Zusätzlich könnten Medikamente zur Strahlensensitivierung genutzt werden, um die mittlere Methylierung nach Therapie zu analysieren [29]. Bei positiven Ergebnissen würden *in-vivo* Experimente notwendig sein, um dieses Phänomen beispielsweise in Mäusen zu untersuchen.
Außerdem fehlten für eine Korrelation der mittleren Methylierung mit dem Ansprechen auf TMZ Zelllinien, die sensitiv auf TMZ ansprachen. Eine Analyse weiterer Zelllinien könnte in Zukunft zeigen, ob auch eine erhöhte zelluläre TMZ-Empfindlichkeit für die Beobachtungen in den Patienten und Patientinnen mitverantwortlich sein könnte. Ergänzt werden müssten diese Arbeiten durch die Analyse entsprechender Patientenkohorten.

2. Abkürzungsverzeichnis

Tabelle 6: Liste der Abkürzungen

Abkürzung	Bedeutung
CpG	Cytosin followed by Guanin
DKFZ	Deutsches Krebsforschungszentrum
DNA	Desoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
GTR	Gross Total Resection
MES	Mesenchymal
MGMT	O6-Methylguanin-DNA Methyltransferase
OS	Overall Survival
PDGFRA	Platelet-derived growth factor receptor alpha
PR	Partiale Resektion
PTK	Protein Tyrosin Kinasen
STK	Serin/Threonin Kinasen
RTK	Receptor tyrosine kinase
TMZ	Temozolomid
WHO	World Health Organization
ZL	Zelllinien
ZNS	Zentrales Nervensystem

3. Publikation 1

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Mean global DNA methylation serves as independent prognostic marker in IDH-wildtype glioblastoma

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Abstract

Background. The IDH-wildtype glioblastoma (GBM) patients have a devastating prognosis. Here, we analyzed the potential prognostic value of global DNA methylation of the tumors.

Methods. DNA methylation of 492 primary samples and 31 relapsed samples, each treated with combination therapy, and of 148 primary samples treated with radiation alone were compared with patient survival. We determined the mean methylation values and estimated the immune cell infiltration from the methylation data. Moreover, the mean global DNA methylation of 23 GBM cell lines was profiled and correlated to their cellular radiosensitivity as measured by colony formation assay.

Results. High mean DNA methylation levels correlated with improved survival, which was independent from known risk factors (*MGMT* promoter methylation, age, extent of resection; $P = 0.009$) and methylation subgroups. Notably, this correlation was also independent of immune cell infiltration, as higher number of immune cells indeed was associated with significantly better OS but lower mean methylation. Radiosensitive GBM cell lines had a significantly higher mean methylation than resistant lines ($P = 0.007$), and improved OS of patients treated with radiotherapy alone was also associated with higher DNA methylation ($P = 0.002$). Furthermore, specimens of relapsed GBM revealed a significantly lower mean DNA methylation compared to the matching primary tumor samples ($P = 0.041$).

Conclusions. Our results indicate that mean global DNA methylation is independently associated with outcome in glioblastoma.The data also suggest that a higher DNA methylation is associated with better radiotherapy response and less aggressive phenotype, both of which presumably contribute to the observed correlation with OS.

Key Points

1. GBM patients treated with combination therapy showed a significantly better outcome if tumors showed a higher DNA methylation.
2. Higher DNA methylation was associated with higher radiotherapy response in GBM patients and higher radiosensitivity of cell lines.

Glioblastoma multiforme, IDH-wildtype (GBM) is the most common malignant brain tumor in adults and is classified as CNS WHO grade 4.[1]The median age of GBM patients is 64 years and the female-to-male ratio is 1:1.6.[2,3] Patients, who do not receive additional therapy after resection have a median overall survival (OS) of less than 6 months after diagnosis.[2] Standard therapy, consisting of surgery and adjuvant radiochemotherapy,[4] increases the OS to 12 to 15 months.[2] However, these values vary individually, with 15% of the patients surviving only 6 months or less after diagnosis and 20% surviving longer than

Importance of the Study

Patients with GBM are typically treated with a combined radiochemotherapy, but median overall survival is still below two years after diagnosis. Additional prognostic markers are urgently needed to better understand disease outcome and predict response to therapy. Robust analytical methods, such as global DNA methylation, are suitable for this purpose in order to improve the therapy of GBM patients in the future. Here, we identified mean global DNA methylation as a prognostic marker, which was associated with higher radiosensitivity.

2 years.[3] Known prognostic markers are age and extent of resection.[5,6] Furthermore, *MGMT* (O6-methylguanine–DNA methyltransferase) promoter methylation status is beneficial for survival by increasing the response to temozolomide-based chemotherapy.[5,7,8] Nevertheless, further clinical and molecular markers are urgently needed to stratify patients and to predict response to therapy.

In general, DNA methylation data can be used through cluster analysis of methylation sites or through the identification of prognostic markers based on copy number profiles inferred from methylation data. In the last years, prognostic markers were examined in order to distinguish tumors according to their aggressiveness and to better predict their response to therapy. These efforts have also included genome-wide DNA methylation by using Illumina arrays,[9–11] and the analysis of such genome-wide DNA methylation signatures has led to the separation of more than 80 tumor entities of the central nervous system. For GBM, several methylation subgroups have been described.[12] The three most frequent subgroups of GBM are mesenchymal (MES), receptor tyrosine kinase I (RTK I), and receptor tyrosine kinase II (RTK II),[12–14] which account for about 90% of the cases and are associated with an equally poor survival.[13,15] RTK II [enriched for epidermal growth factor receptor (*EGFR*) gene amplifications] occurs most frequently (40%–50%), followed by MES (20%–45%) [enriched for neurofibromin-1 (*NF1*) aberrations].[14,16] RTK I, which is enriched for platelet-derived growth factor receptor-alpha (*PDGFRA*) gene amplifications, is the least common of the three subgroups (about 20%).[14] Despite the success of the epigenetic analyses in molecular neuropathology, a prognostic value, which is evident for multiple entities, such as ependymoma and medulloblastoma, has not been identified for GBM so far.[17,18]

Using a large cohort of 492 GBM patients homogeneously treated according to standard therapy, we here propose how to make the methylome data usable for robust survival prognosis.

Material and Methods

Patient Cohort

Three different cohorts were analyzed, including a total of 645 primary and 31 relapsed GBM samples. The first patient cohort consists of 492 GBM patients from three German hospitals (Charité Berlin, University Hospital of Frankfurt, University Medical Center Hamburg-Eppendorf) and previously published data (The Cancer Genome Atlas (TCGA), Gene Expression Omnibus database (GEO) under the accession numbers GSE60274, and GSE195640).[14] All samples were classified as GBM that had the highest score as GBM on the Heidelberg brain tumor classifier (v11.6). Age, survival, extent of resection, DNA methylation profiles, and therapy details were available for most patients. The extent of resection (EOR) was divided into gross total resection (GTR), near GTR, and partial resection or stereotactic biopsy. The complete removal or removal of more than 90% of contrast-enhancing parts was defined as a GTR or near GTR, whereas a resection of less than 90% was defined as partial resection or biopsy. The EOR of contrast-enhancing parts was evaluated by MRI performed up to 48 h after surgery.[5,6] Only patients treated with a combination of radiation and temozolomide-based chemotherapy (Stupp regimen) were included in this first cohort. The second cohort, which was derived from previously published data (GEO under the accession numbers GSE60274, GSE195684, and GSE195640)[14] includes only patients, who did not receive chemotherapy ($n = 148$). The third cohort includes 31 patients who had a second resection because of a recurrence, and the methylome was analyzed for both of their tumors. For this study, 30 patients were treated by the Stupp regimen in the first-line therapy, and one patient was treated with temozolomide-based chemotherapy alone. The use of biopsy-specimens for research was approved and in accordance with local ethical standards and regulations at the University Medical Center Hamburg-Eppendorf (PV4904).

DNA Methylation Profiling

Extracted tumor DNA was analyzed for genome-wide DNA methylation patterns using the Illumina 450k array or Illumina EPIC (850k) array. All data are available at https://www.ncbi.nlm.nih.gov/geo/ under the accession number GEO240704. The DNA methylation data obtained were further processed using previously described approaches.[12] The analysis and visualization of the DNA methylation data were performed with R (version 3.6.0). The IDAT files of the two arrays used (Infinium HumanMethylation450 BeadChip and Infinium MethylationEPIC BeadChip) were processed and combined using minfi Bioconductor package v3.9. Probes located on two nucleotides, containing a single-nucleotide polymorphism, or located on the X or Y chromosome were excluded. The preprocess noob function of the minfi package was employed, and beta values were calculated. For the batch effect correction regarding the array type, the package limma (v3.40.0) was used. To verify the analyses based on the minfi, the sesame package (v1.18.4)

was also used to preprocess the IDAT files. The delta beta value described the difference in mean methylation of both groups. Usually, a delta beta value of at least 0.2 is used. Mean DNA methylation was determined from the mean of all beta values per sample.

The package DIMEimmune was used to estimate the number of $CD4^+$ and $CD8^+$T cells and the number of tumor-infiltrating lymphocytes (TILs).[19] Analysis of immune cell infiltrates was performed using the DIME (Differential Methylation Analysis for Immune Cell Estimation) score described in Safaei et al.[19] For the analysis of the other cell populations, EpiDISH with the reference file of Grabovska et al. was used (v2.16.0).[20] The proportion of myeloid cells was calculated by summing all proportions of eosinophils, neutrophils, and monocytes.

To calculate and visualize the copy number aberrations per sample, the conumee package (v1.34.0) was used. The modified total aberration index (tai) was calculated with the CINmetrics package (v0.1.0). For the tai, the length of a segment was multiplied by the segmentation mean if there was a gain or loss in that segment (threshold: ±0.1). Then, these products were added for all segments and divided by the sum of the segment lengths, describing the tai per sample. If tai was equal to 0, the genome was balanced.

Statistical Analysis

The calculation of the best cut-off was performed with the function maxstat.test from the package maxstat (v0.7-25), the log-rank test by using the ranked statistics and the method Lau94 were used for the calculation of the best cut-off. The package ggplot (v3.4.0) was used to represent the data by boxplots. For survival analysis, Kaplan–Meier plots were generated with the packages survminer (v0.4.4) and survival (v3.4-0). The groups were compared using the log-rank test. The multivariate analysis was performed with the finalfit package (version 1.0.5).

Cell Culture

The GBM cell lines LN229, U87MG, LN827, LN71, U343, and Cas-1 were cultured in DMEM (Sigma–Aldrich) supplemented with 10% FCS (Biochrome), 2 mM L-glutamine, and 1 mM sodium pyruvate (Sigma–Aldrich); DKMGvIII- cells were cultured in RPMI (10% heat inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate) while BS153vIII- cells were cultured in DMEM (10% heat inactivated FCS, 2 mM L-glutamine and 1 mM sodium pyruvate). All cells were cultured at 37°C, 5% CO_2, and 100% humidification and were authenticated using short tandem repeat (STR) profiling by Eurofins.

Cell Survival and X-Irradiation

Cell survival after exposure to X-ray was analyzed by colony formation assay. Two hundred and fifty to 350 cells were placed in six-well plates and irradiated 24 h later (Gulmay RS225; Gulmay Medical Ltd.; 200 kv, 15 mA, 0.8 mm Be + 0.5 mm Cu filtering; dose rate of 1.2 Gy/min). Twenty-four hours after the treatment, the medium was replaced. Only for BS153, AmnioMax C-100 Basal Medium (Life Technologies) containing 10% FCS and C-supplement (Life Technologies) was used instead of the usual medium to improve colony formation. Colonies were allowed to grow for 1.5 to 3 weeks depending on the cell line and irradiation dose. Afterward, the colonies were fixed with 70% ethanol and stained with crystal violet. Colonies with at least 50 cells are counted. Cell survival analysis was performed in PRISM (v6.07). We classified the cell lines according to their cell survival at an irradiation dose of 4 Gy. For this purpose, we chose the best cut-off value for each cohort using their mean global DNA methylation.

Results

Patient Characteristics Are Representative in Demographics and Overall Survival

The aim of this study was to analyze the prognostic value of methylome data for GBM patients. Therefore, we first collected and analyzed DNA methylation and OS data from 492 GBM patients, who were homogeneously treated with standard combination therapy (Table S1). Cohort characteristics including the known prognostic factors age, *MGMT* promoter methylation, and extent of resection are displayed in Figure 1A. The mean age was 59.5 years with a female-to-male ratio of 1:1.6, while *MGMT* promoter methylation was present in 47.4% of the cases. Information on the extent of resection was available for 315 patients, with most tumors being resected (84.8%), with the extent of resection ranging from gross total resection (GTR, 37.8%), to near GTR (26.7%) to partial resection (20.3%). The median OS was 15.8 months, with 168 cases being censored (Figure 1B). Twenty percent of the patients survived 7.5 months or less, while another 20% survived at least 35 months. In accordance with the literature, age, *MGMT* promoter methylation as well as the extent of resection were independent prognostic factors (Supplementary Figure S1).

Mean DNA Methylation Is Significantly Different in Long and Short Surviving Patients

Next, we stratified the cohort into long and short surviving patients based on the median OS of 15.8 months. If patients were censored before 15.8 months of survival, they were excluded ($n = 106$), as it was unknown, to which group they would be definitively assigned. Within the remaining 386 samples, we did not detect significant differences in the methylation of single CpG sites and, therefore, failed to identify a methylation signature that is associated with better or worse survival of GBM patients (Supplementary Tables S2 and S3).[21]

To further investigate epigenetic prognostic markers of survival, we calculated the mean methylation of each sample and the median methylation of the entire cohort, which was 0.49 (Figure 2A). Dividing the cohort according to this median methylation, a significant difference in OS was detected (Figure 2B). A higher level of mean DNA methylation was associated with a significantly better OS (methylation > 0.49, median OS: 18.0 months), while

A

Category		
Age	median (range) [years]	60 (27–86)
Sex	female	188 [38.2%]
	male	304 [61.8%]
MGMT promoter methylation	methylated	234 [47.6%]
	unmethylated	258 [52.4%]
Methylation subgroups	MES	158 [32.1%]
	RTK I	120 [24.4%]
	RTK II	214 [43.5%]
Extent of resection	GTR	119 [37.8%]
	Near GTR	84 [26.7%]
	Partial	64 [20.3%]
	Biopsy	48 [15.2%]
Origin	UKE, Hamburg	189 [38.4%]
	Charité, Berlin	60 [12.2%]
	Frankfurt	67 [13.6%]
	TCGA	86 [17.5%]
	GEO, GSE 60274	21 [4.3%]
	GEO, GSE195640	69 [14.0%]

B

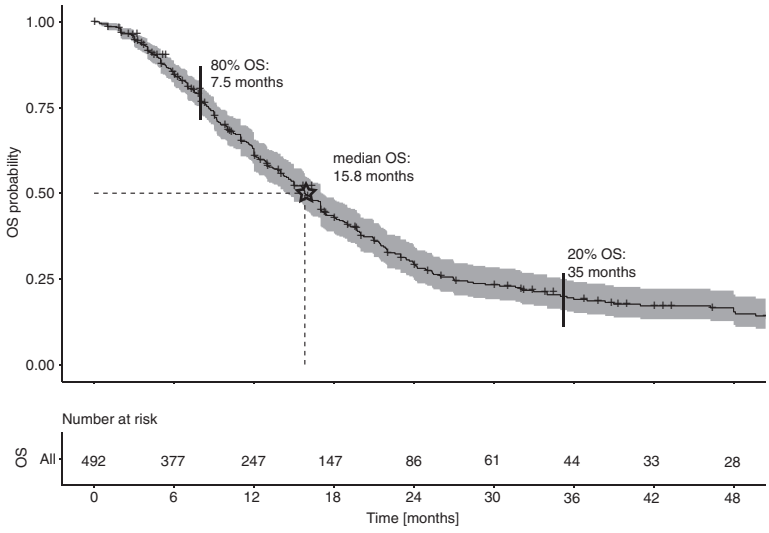

Figure 1. Overview of the GBM patient cohort. Analysis based on (A) clinical data and (B) Kaplan–Meier plot with the median overall survival marked with an asterisk.

a lower methylation was associated with a significantly worse OS (median OS: 13.0 months; $P = 0.007$). Further analysis using the best cut-off with the ranked statistics for the mean methylation (beta value = 0.458) identified a small group of patients, whose tumors had very low mean DNA methylation ($n = 73$) and showed a highly significant lower OS ($P < 0.001$) with a median OS of 9.2 months compared to 17.0 months (Figure 2C, D). Subsequent multivariate analysis showed that mean methylation is an additional prognostic factor, independent of age, *MGMT* promoter methylation, and extent of resection (HR: 1.50; 95% CI: 1.11–2.04; $P = 0.009$, Figure 2E).

Next, we analyzed a potential bias due to the extent of resection. However, both groups (GTR/near GTR and partial resection/biopsy) showed comparable levels of mean methylation (Supplementary Figure S2A). Moreover, the level of mean methylation was a significant prognostic marker in both groups (Supplementary Figure S2B–C). Furthermore, we investigated the influence of copy number aberrations on the mean methylation by calculating the modified total aberration index (tai). However, there was no significant difference in the survival after splitting the cohort at the best cut-off of the tai (Supplementary Figure S2D). Also, tai scores were not significantly different with respect to mean methylation using either minfi or sesame for preprocessing (Supplementary Figure S2E, F). Most of the samples had a negative tai score implying a negative balance regarding copy number aberrations. This observation was confirmed in cumulative plots of the high or low methylated samples (Supplementary Figure S2G, H). Therefore, copy number aberrations did not show any correlation to the mean methylation in our cohort.

We next classified all cases using the Heidelberg brain tumor classifier. As expected from the literature,[13,15] there was no significant difference in patient survival between mesenchymal (MES), RTK I, and RTK II GBM subgroup (Supplementary Figure S3A). When analyzing the median methylation of the three GBM subgroups, we observed that RTK I tumors had a significantly lower mean methylation level than MES or RTK II tumors (Supplementary Figure S3B; $P < 0.001$). Still, a high mean methylation was correlated with better survival in patients with RTK I and RTK II GBM (best cut-off; Supplementary Figure S3C–E). When including the GBM subgroup into the multivariate analysis, the mean methylation still remained an independent prognostic factor (Supplementary Figure S3F).

In order to analyze whether mean DNA methylation is generally correlated with tumor aggressiveness, we employed a reference set including 91 brain tumor and control methylation classes[12] and compared the mean methylation of the different (sub-)entities (Supplementary Figure S4). However, comparatively high methylation was also observed in very aggressive tumors, such as atypical teratoid/rhabdoid tumors (ATRT),[22] and less deadly brain tumors, such as pituitary adenomas may also have lower mean DNA methylation levels. These findings demonstrate that a high-mean methylation is not generally related to a better outcome.

Since we have shown that mean global DNA methylation is an independent prognostic marker for standard-treated GBM, we next combined all independent prognostic markers to calculate three risk groups considering only patients with available information for all prognostic markers ($n = 315$, Figure 2F). For this, we defined all combinations where the Kaplan–Meier plots showed no significant difference between them, as a specific risk group. Risk group 1, which had the best survival, included all patients, who had no negative prognostic marker (median OS: 39.0 months; Figure 2G). The second risk group

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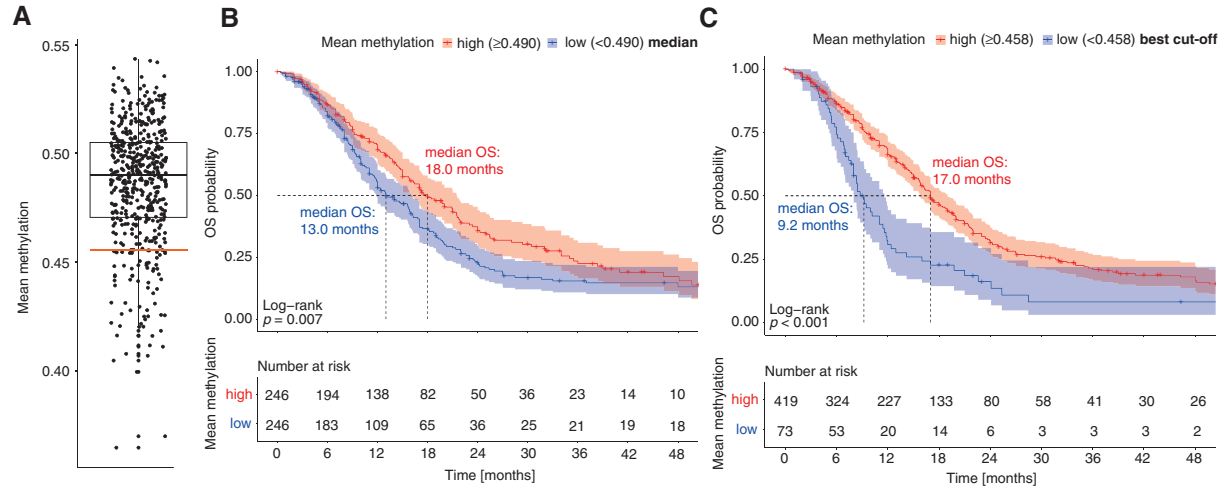

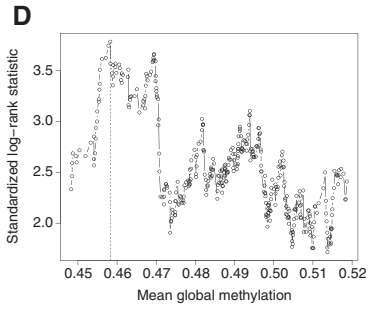

E

	levels	all	HR (univariable)	HR (multivariable)
Age (10 years interval)	mean (SD)	59.5 (10.5)	1.22 (1.10–1.34, p < 0.001)	1.34(1.10–1.48, p = 0.001)
MGMT promoter methylation	methylated	234 (47.6)	-	-
	unmethylated	258 (52.4)	2.01 (1.60–2.52, p < 0.001)	2.01 (1.49–2.72, p < 0.001)
Mean methylation	high	246 (50.0)	-	-
	low	246 (50.0)	1.35 (1.09–1.69, p = 0.007)	1.50 (1.11–2.04, p = 0.009)
Extent of resection		112 (35.6)	-	-
		203 (64.4)	0.38 (0.28–0.52, p < 0.001)	0.36 (0.26–0.49, p < 0.001)

F

#	Age	*MGMT*	EOR	MM	n	events	Median OS	Risk group
Zero prognostic factors								
1					61	28	39.0	1
One prognostic factor								
2					28	17	24.9	2
3					58	31	19.7	2
4					21	12	23.0	2
5					4	2	8.3	2
Two prognostic factors								
6					25	16	13.0	2
7					8	7	19.8	2
8					32	19	11.0	3
9					5	4	12.9	3
10					19	9	10.0	3
11					9	5	11.8	3
Three prognostic factors								
12					7	6	11.4	3
13					5	5	7.4	3
14					10	9	7.4	3
15					17	12	8.0	3
Four prognostic factors								
16					6	5	5.4	3

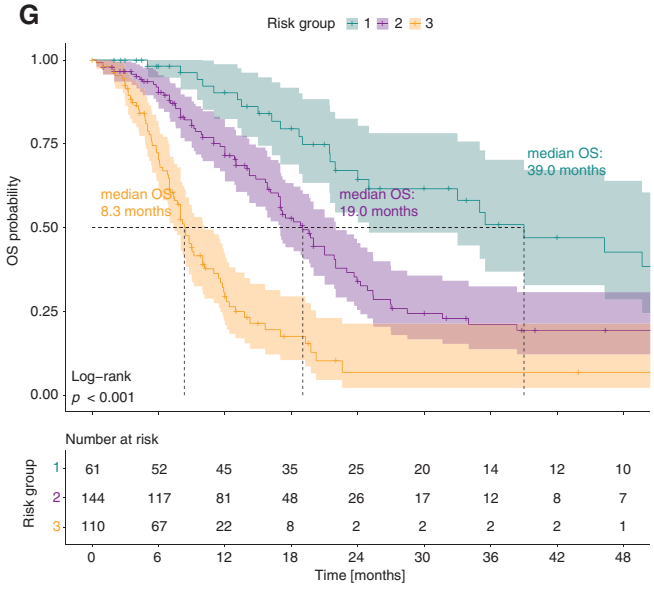

Figure 2. Analysis of mean methylation of 492 GBM patients. (A) Distribution of mean methylation marking the median (black) and best cutoff (orange). (B, C) Kaplan–Meier curves after division of the cohort at the (B) median mean methylation level and (C) best cutoff using the (D) calculated ranked statistics. (E) Multivariate analysis of mean methylation in combination with known prognostic factors. (F) Combinations of all prognostic markers [Age (threshold: >65 years), EOR (extent of resection), *MGMT* promoter methylation, MM (mean DNA methylation)] colored in green (negative prognostic marker) and orange (positive) and (G) Kaplan–Meier plots of three risk groups (1: orange, 2: purple, 3: green).

described all patients with either one prognostic factor or one of the combinations high age + methylated *MGMT* promoter or high age + low mean methylation (median OS: 19.0 months). The worst survival was observed in the risk group 3 (median OS: 8.3 months). It included all patients with at least three prognostic factors, any combination with a low extent of resection or the combination of unmethylated *MGMT* promoter + low mean methylation. All three risk groups differed significantly in OS ($P < 0.001$).

Immune Cell Infiltration Does Not Explain the Correlation From OS and Mean Methylation

Since differences in OS can arise from differences in immune cell infiltration with more infiltrating immune cells reportedly being associated with better OS,[23] we next analyzed whether differences in mean methylation are associated with the number of infiltrating immune cells. To this end, we used a deconvolution algorithm (DIME Immune Score[19]) to estimate the number of CD4+, CD8+, and tumor infiltrating lymphocyte (TIL) cells in our series of GBM. In line with the literature, we observed a longer OS of GBM patients with higher DIME scores (best cutoff of for each DIME score: CD4+: –0.026; CD8+: 0.448; TIL: 0.956; Figure 3A–C). Importantly, the DIME scores for all three immune cell infiltrates were significantly higher in the low methylated group of GBM using the best cutoff as in Figure 2C than in the higher methylated tumor samples (Figure 3D–F; CD4+: $P < 0.001$; CD8+: $P = 0.003$; TIL: $P = 0.024$). To further investigate the influence of distinct cell populations on mean methylation, we additionally used EpiDISH to analyze the populations of myeloid cells, in particular monocytes, neurons, and glial cells with respect to mean methylation (Supplementary Figure S5). Indeed, samples with high methylation showed significantly more myeloid cells and monocytes (Supplementary Figure S5A, B), whereas numbers of neurons and glia cells were comparable in GBMs with low- or high-mean methylation. Looking at the GBM subgroups, only MES GBM had a high proportion of myeloid cells, whereas RTK-II-GBM did not (Supplementary Figure S5E). However, since mean methylation was not significantly different in MES and RTK II tumors, the proportion of myeloid cells did not appear to be the only reason for high mean methylation. Therefore, our data suggest that infiltrating immune cells were not causative for the better survival of patients with increased methylated GBM.

Higher Mean DNA Methylation Is Associated With Higher Radiosensitivity

All patients analyzed so far had received standard therapy including radiotherapy and temozolomide (TMZ). To investigate a correlation of mean methylation to therapy, we

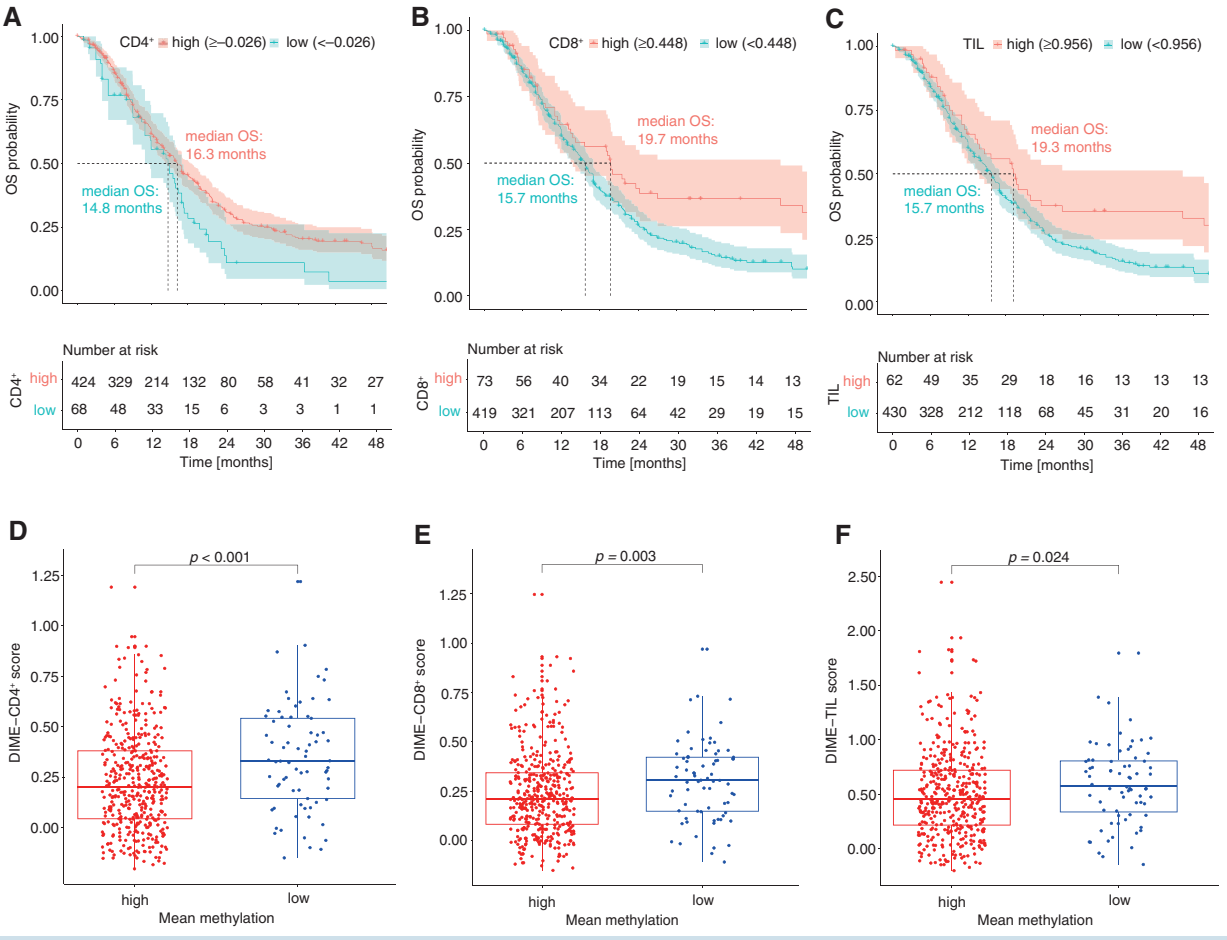

Figure 3. Correlation of CD4+, CD8+, and TIL immune cell counts to mean methylation. (A–C) Kaplan–Meier curves dividing the cohort at the best cutoff of the respective DIME scores and DIME score of (D) CD4+, (E) CD8+, and (F) TIL for low- and high-methylated tumor samples.

performed the analyses with irradiation as monotherapy. To this end, we determined the cellular radiosensitivity of a panel of commercially available GBM cell lines by colony formation assay and stratified them by the survival fraction at 4 Gy (SF4) in radiosensitive and radioresistant cell lines (Figure 4A). DNA methylation was analyzed for these, and an additional panel of 15 stem-like cell lines with published SF4 data.[24] The latter were also divided in radiosensitive and radioresistant cell lines as shown in Figure 4B. The analysis of cumulative copy number variations within these cell lines did not show any obvious differences that could cause differences in radiosensitivity (Supplementary Figure SF6). However, the combined analysis of SF4 and mean methylation for both cohorts revealed a significantly higher mean methylation for radiosensitive cell lines compared to radioresistant cell lines (Figure 4C; $P = 0.007$). This suggested that increased cellular radiosensitivity might be a cause for improved survival of patients with highly methylated GBM. To support this hypothesis, we correlated radiotherapy response with mean DNA methylation in 148 patients, who received radiotherapy but no TMZ (Table S4). Patients divided at the best cut-off with higher mean methylation survived significantly longer (median OS: 12.6 months) than patients with lower mean methylation (median OS: 9.4 months; $P = 0.029$; Figure 4D). By defining two risk groups based on age, *MGMT* promoter methylation, and mean DNA methylation the difference in OS even ranged from 8.4 to 12.5 months (Figure 4E, F). With low-mean methylation of the tumors, patients survived significantly shorter and are classified in risk group 2 (combination 4 and 6-8). Patients with at least two negative prognostic markers due to their advanced age and low-mean methylation showed the shortest median survival (median OS (combination 6): 7.6 months and median OS (combination 8): 7.2 months). The risk group 1 showed a significantly longer OS (median OS: 12.5 months) than the risk group 2 (median OS: 8.4 months; $P = 0.002$; Figure 4F).

Changes in Mean DNA Methylation During Tumor Recurrence

Finally, we tested if mean methylation might also be a general marker for a lower relapse risk. Since recurrent GBM tumors have a more aggressive phenotype than primary tumors,[25] we analyzed changes in the mean DNA methylation during first and second resection in a cohort of 31 tumor pairs (Table S5). The tumor pairs indeed

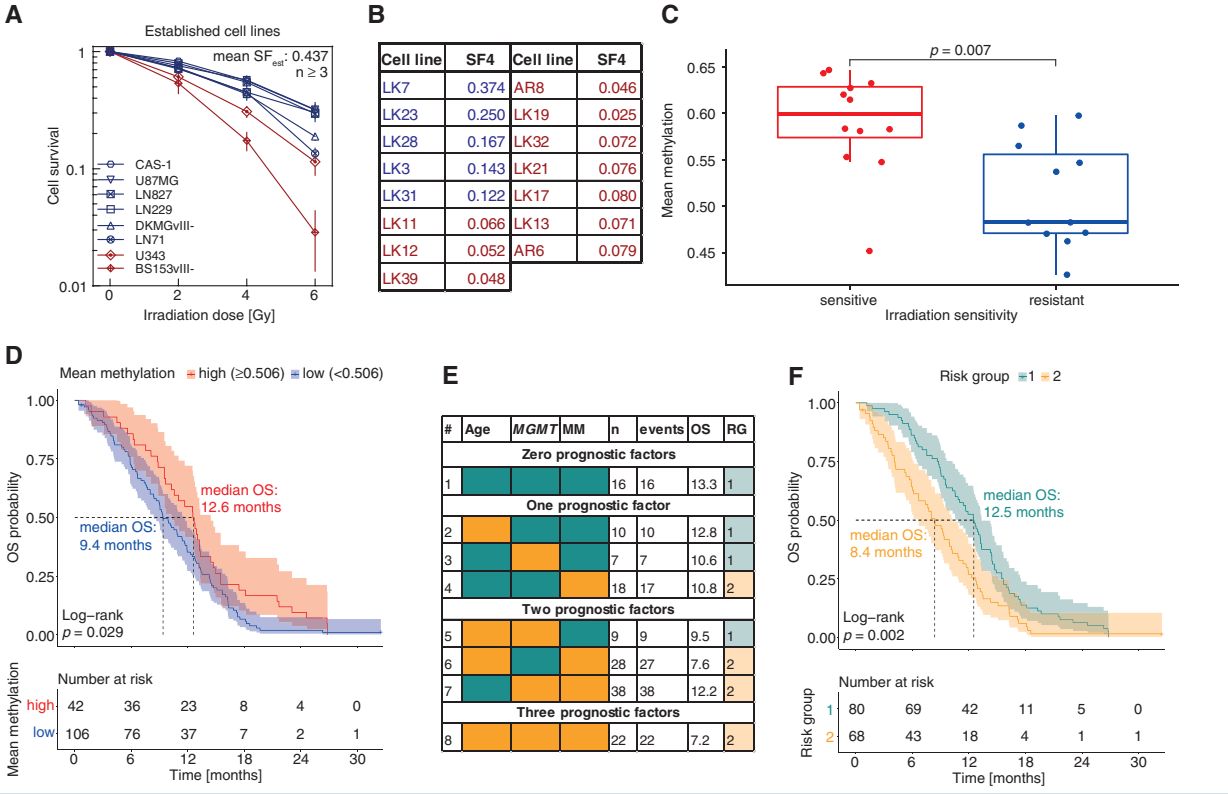

Figure 4. Radiosensitivity (red: radiosensitive, blue: radio resistant) of GBM (A, B) cell lines. Dose response curves of eight established GBM cell lines (surviving fraction at 4 Gy: SF4$_{est}$). (B) SF4 of 15 GBM stem-like cells.[24] (C) Combination of both cohorts and correlation of the mean methylation with SF4 (radiosensitive: red, radioresistant: blue). (D–F) Patient data. (D) Survival of patients treated with irradiation only (148) analyzed by Kaplan–Meier analysis. The curves are divided at the best cutoff (higher methylation: red, lower methylation: blue). (E) Combinations of all prognostic markers [Age, EOR (extent of resection), *MGMT* promoter methylation, MM (mean DNA methylation)] colored in green (negative prognostic marker) and orange (positive) and (F) Kaplan–Meier analysis of two risk groups (1: orange, 2: green).

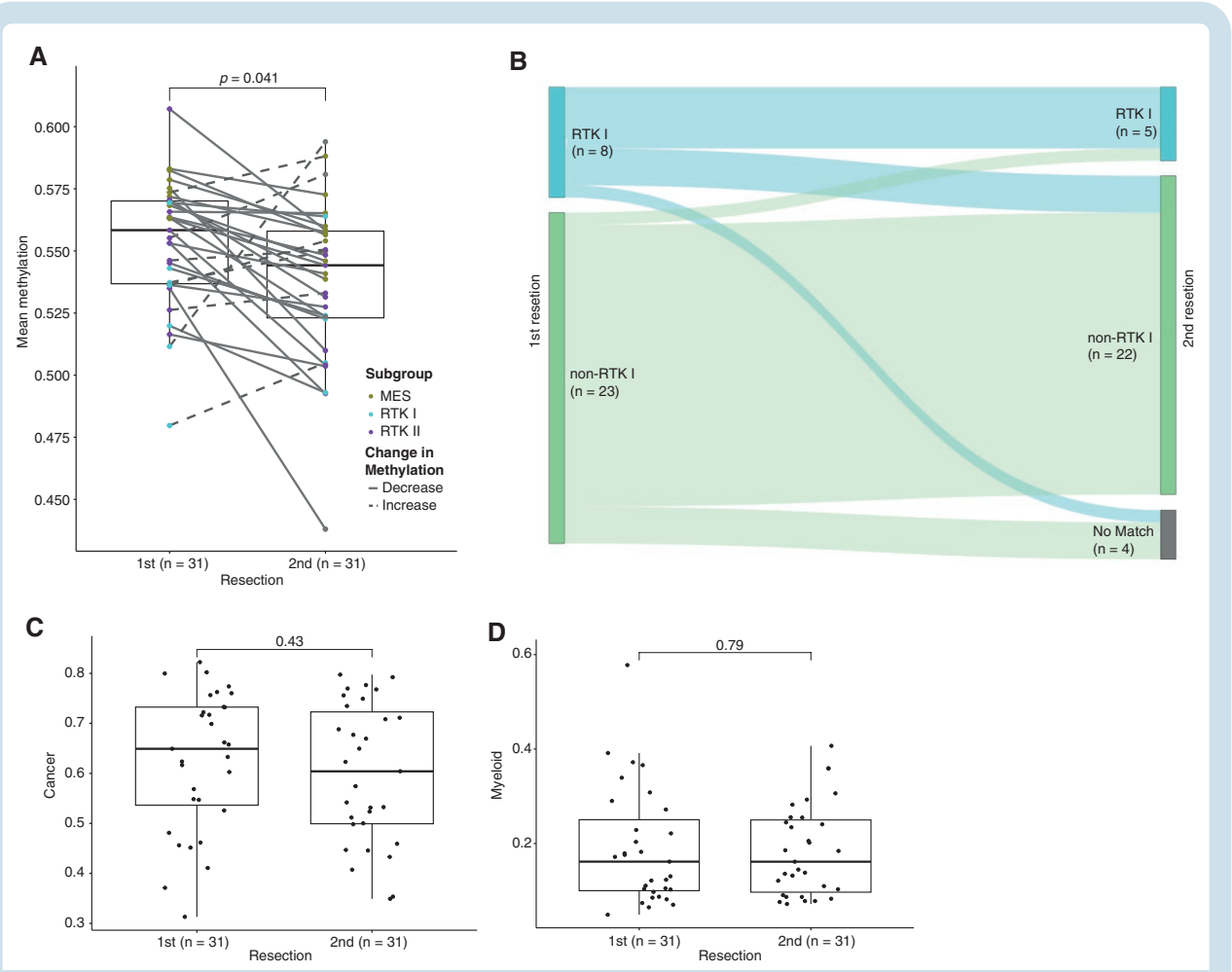

Figure 5. Changes in mean DNA methylation during disease progression. (A) Analysis of recurrence samples (31) versus matching primary tumor (31) regarding mean DNA methylation and (B) subgroup switching between RTK I and non-RTK I. Analysis of the cell populations in the recurrence samples versus primary tumor of (C) cancer cells and (D) myeloid cells.

showed a difference in mean methylation between the first and second resection (Figure 5A) with a significantly lower mean methylation in the re-resected samples ($P = 0.041$). Only eight tumors had higher mean methylation at the second compared to the first resection. Because RTK I tumors have a significantly lower mean methylation level than the other two subgroups (Supplementary Figure S3B), and it is known that a change of the tumor subgroup can occur during therapy,[16] we determined possible subgroup switches (Figure 5B). We identified four RTK I tumors which changed the subgroup between resections, while there was only one non-RTK I tumor that underwent a subgroup change from non-RTK I to RTK I subtype. This indicates that lower mean methylation is not caused by a subgroup switch but is associated with a more aggressive tumor phenotype. Furthermore, using EpiDISH as method for deconvolution, we analyzed and compared the tumor cell content and proportion of myeloid cells in the samples from the two resections. There was no significant difference between the first and second resection in terms of the cell populations examined in the samples (Figure 5C, D). Therefore, lower mean methylation cannot be explained by fewer myeloid cells in this cohort either.

Discussion

Fifty percent of glioblastoma patients survive only below 2 years after diagnosis despite intensive therapy. Previously, prediction of response to temozolomide-based chemotherapy was possible by analysis of *MGMT* promoter methylation. In addition, prediction of patient survival is also possible to a limited degree based on patient age and extent of resection. With this study, we demonstrate the prognostic value of genome wide DNA methylation for GBM. In contrast to the GBM subgroup classification achieved by distinct methylation patterns—which have no clear prognostic value—the prognostic impact lies in the mean methylation of a given tumor. The mean methylation is a simple and robust prognostic marker and was demonstrated to be independent from other prognostic

markers, such as age, *MGMT* promoter methylation, and extent of resection. The combination of all four markers even allows a more precise prediction of OS after standard therapy and a clear stratification of patient subgroups with worst (median OS: 8.3 months), moderate (median OS: 19.0 months), and relatively good prognosis (median OS: 39.0 months).

We tried to determine individual CpG sites, which are informative of patient survival but the methylation of single CpG sites is probably not sufficient to predict OS of GBM patients. It has been described by others that specific differentially methylated CpG sites can be associated with OS.[10] However, this is likely due to a more heterogeneous cohort and patients, who have not received adjuvant radiochemotherapy. The mean age of the 492 patients analyzed in our project reflected the known literature as well as gender distribution, and proportion of *MGMT* promoter methylation.[3,26,27] Therefore, our cohort was not only particularly large and well annotated but also representative of the demographic distribution and survival of glioblastoma patients.

The three methylation subgroups have no significant difference in survival, but in their mean methylation, which has also been described before.[15] Nevertheless, it does not cause the RTK I subgroup with significantly lower methylation to have shorter survival compared to the other two subgroups. In contrast, a combination of all 4 prognostic factors (age, extent of resection, *MGMT* promoter methylation, and mean global DNA methylation) is more critical to predict survival whereby a higher number of risk factors was associated with worse survival.[28] The combinations are also relevant; hence, the OS of elderly patients is better with low mean methylation than poor extent of resection. In addition, the extent of resection in combination with another prognostic marker had a strong influence on OS; therefore, these patients were classified in risk group 3.

To investigate possible causes of mean global DNA methylation as a prognostic marker, we correlated it with the number of immune cells. Analysis of immune cell infiltrates revealed a correlation between mean methylation and DIME scores (Figure 3D–F). However, the scores are not sufficient for a comparable division of the cohort into longer and shorter surviving patients (Figure 3A–C). Mostafa et al. shows, using 51 glioblastoma patients,[23] that an increased number of $CD8^+$ lymphocytes is associated with better OS. We also see that a high number of $CD8^+$ immune cells correlated with a better OS of GBM (Figure 3B). Contrary to those results, Zuo et al. analyzed the TCGA database and showed that a high immune cell count is associated with worse OS in GBM.[29] They examined 46 immune cell types. This clearly indicates that more analyses need to be performed for exploring the correlation between immune cell infiltrates and OS to fully understand the interplay. Our analyses show a significant correlation of OS to immune cell infiltration, with higher immune cell infiltration associated with better survival. The correlations are significant but not as strong as the correlation of mean methylation with survival. Causality of mean methylation due to more immune cells cannot be discerned from our results. In addition, the analysis of cell populations in terms of mean methylation and survival may also be promising, as a higher proportion of myeloids correlates with higher methylation. Nevertheless, a direct causal relationship cannot yet be observed. Moreover, in addition to deconvolution, further analyses on single cell or histological level should be included in the future, as it is known that deconvolution of bulk data have strong limitations in accuracy. The analyses of cell populations in the samples are crucial for further understanding of the biological causality regarding mean methylation. Biological causality also needs further investigation to explain why mean methylation is not relevant in the mesenchymal subset but is in RTK I and RTK II.

Another possible explanation for the correlation of survival and mean methylation is a better response to radiotherapy, meaning that tumor cells with higher mean DNA methylation are more radiosensitive. This is supported by the better survival of patients treated with radiotherapy alone with a higher mean methylation (Figure 4E–F). Although significant, the difference in survival was not particularly large. This could be explained by a poor performance status of at least some patients, since clinical parameters such as age or health status might be crucial reasons why patients were not treated with chemotherapy or standard of care. Since irradiation varied between the two published cohorts (30 × 2 Gy or 10 × 3.4 Gy), it will be important in the future to study a large cohort of patients, who received the same fractionation and dose with respect to mean methylation.

Although higher radiosensitivity might be explained by immunological mechanisms, such as a high number of M2 macrophages,[30] it is more likely that differences between more resistant and more sensitive tumors are due to differences in the cellular radiosensitivity. This hypothesis is supported by the cell line experiments, which show a higher methylation in more radiosensitive cell lines. Cellular radiosensitivity is a highly complex phenotype, regulated not only by gene alternations but also by factors such as protein expression and availability, signal transduction, oxygenation, or chromatin structure.[31,32] The investigation of the relationship between cellular radiosensitivity and mean methylation is, therefore, outside the scope of this project and will be analyzed in a separate future project. Here, isogenetic cell lines with either high- and low-mean methylation will be helpful and are currently under development.

In conclusion, our study shows that survival of GBM patients was correlated with mean global DNA methylation. Patients with higher mean methylation survived longer, so further research is needed to understand the biological background and to provide new therapies to improve survival.

Supplementary material

Supplementary material is available online at *Neuro-Oncology* (https://academic.oup.com/neuro-oncology).

Keywords

DNA methylation | glioblastoma | overall survival | radiotherapy response

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Conflict of interest statement

None declared.

Data availability

The raw methylation data we used are available at The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus database (GEO) under the accession numbers GSE60274, GSE195640, GSE60274, GSE195684, and GSE195640.

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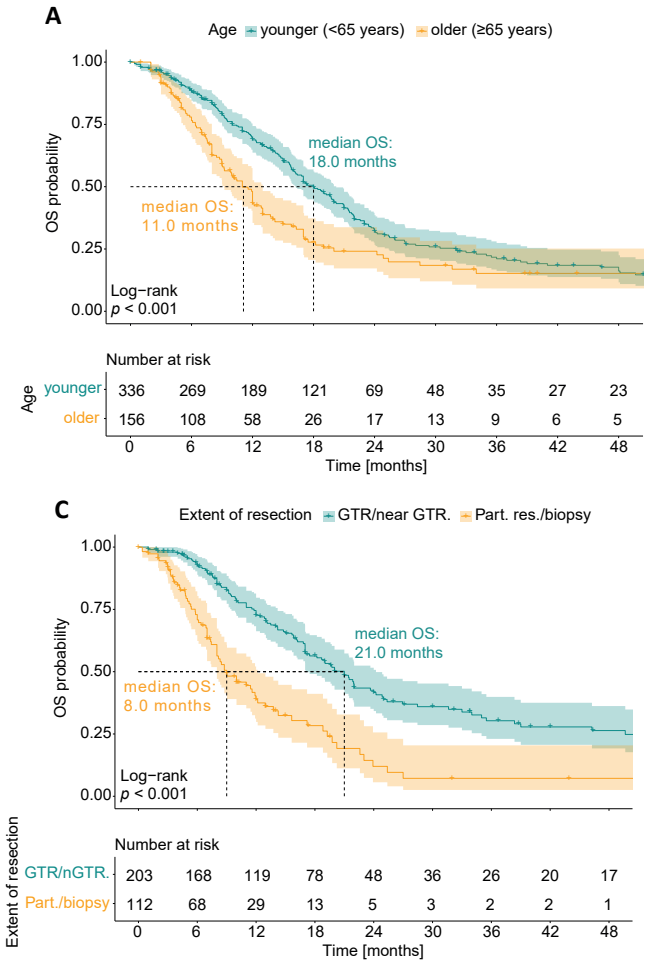
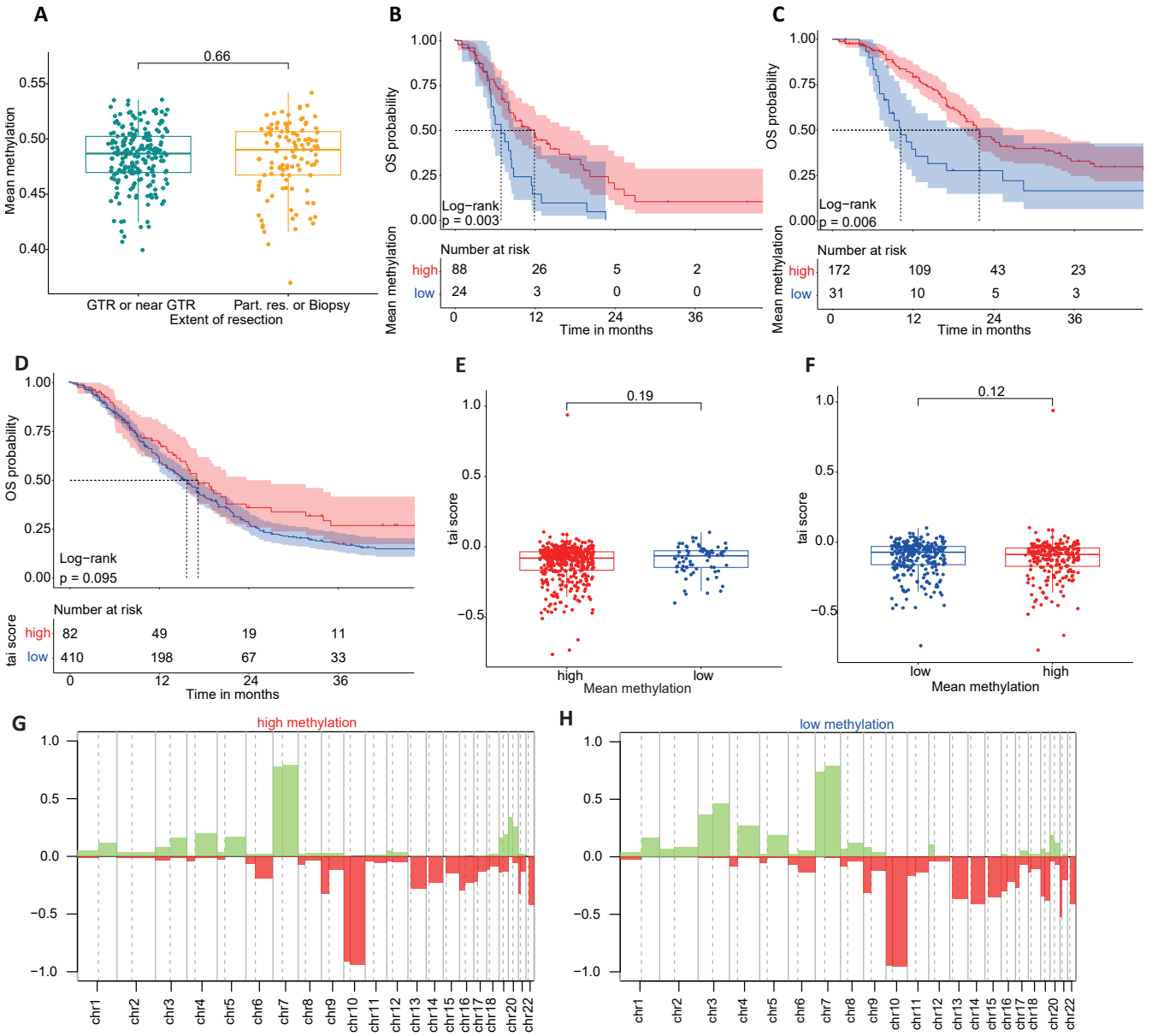


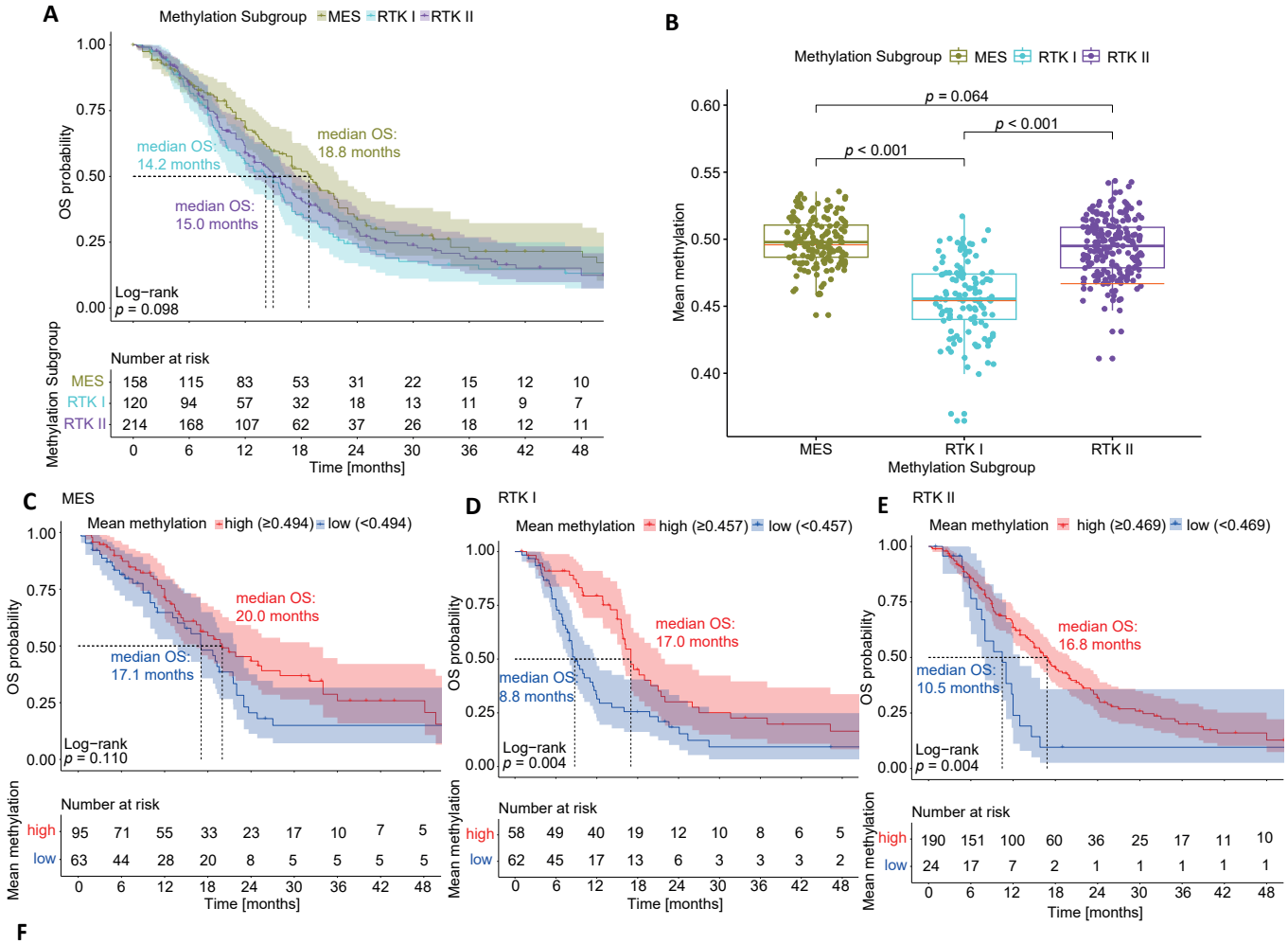
D

	levels	all	HR (multivariable)
Age (10 years interval)	mean (SD)	59.5 (10.5)	1.03 (1.01-1.04, p=0.001)
MGMT promoter methylation	methylated	234 (47.6)	-
	unmethylated	258 (52.4)	2.00 (1.48-2.71, p<0.001)
Extent of resection	partial res. or biopsy	112 (35.6)	-
	GTR or near GTR	203 (64.4)	0.38 (0.28-0.52, p<0.001)

Supplementary Figure 1: Analysis of prognostic factors of n=492 patients. Correlation of OS to all known prognostic factors (**A** age, **B** *MGMT* promoter methylation, **C** extent of resection (GTR: gross total resection, nGTR: near gross total resection, part. res.: partial resection)). **D** Multivariate analysis of known prognostic factors (age, *MGMT* promoter methylation, extent of resection).



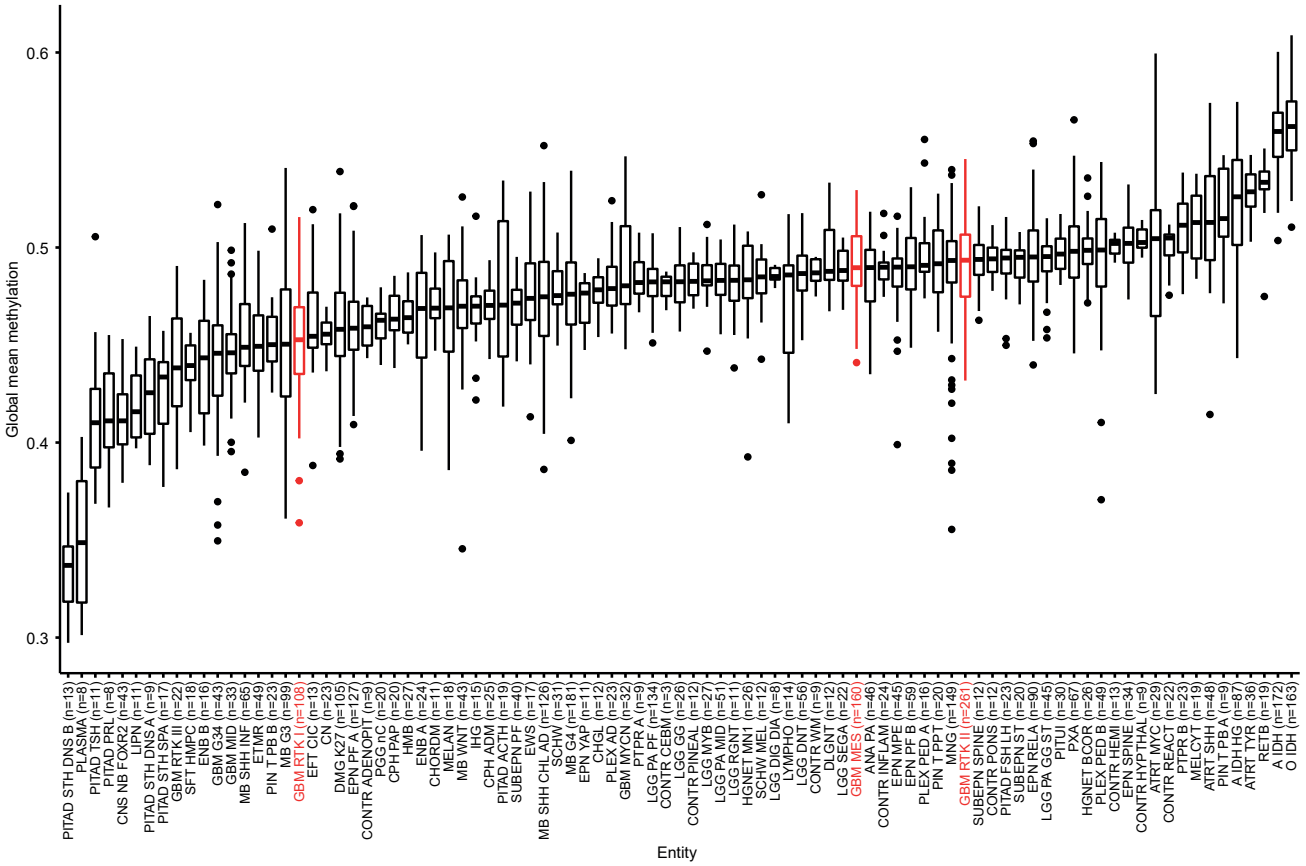
Supplementary Figure 2: Analysis of extent of resection and copy number variations regarding the mean methylation. **A** Comparison of mean methylation with respect to mean methylation, Kaplan-Meier plots of **B** partial resection/biopsy samples and **C** GTR/near GTR samples. **D-F** Analysis of tai score regarding mean methylation using **D** the best cut-off and analyze the OS, analysis of the tai score with respect to mean methylation using **E** minfi and **F** sesame. Cumulative copy number plots of samples with a **G** high methylation and **H** low methylation using minfi and the best cut-off.



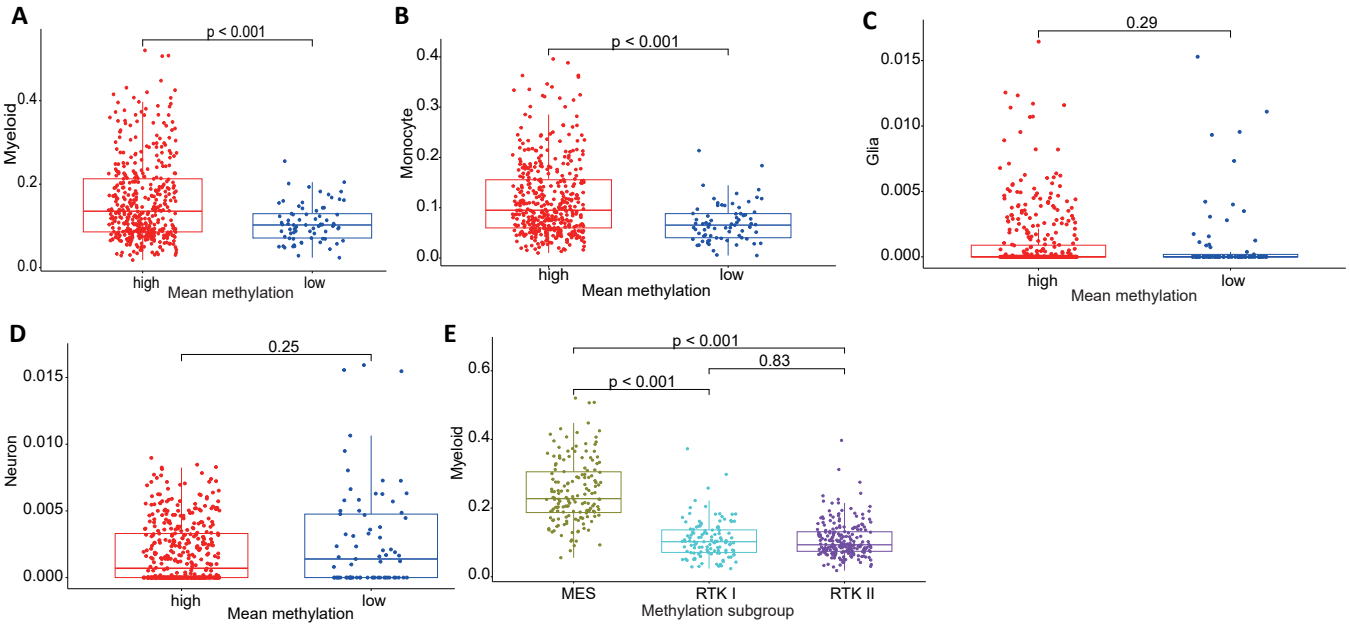
F

	levels	all	HR (univariable)	HR (multivariable)
Age (10 years interval)	mean (SD)	59.5 (10.5)	1.22 (1.10-1.34, p<0.001)	1.34 (1.10-1.48, p=0.001)
MGMT promoter methylation	methylated	234 (47.6)	-	-
	unmethylated	258 (52.4)	2.01 (1.60-2.52, p<0.001)	2.03 (1.50-2.75, p<0.001)
Mean methylation	high	246 (50.0)	-	-
	low	246 (50.0)	1.35 (1.09-1.69, p=0.007)	1.44 (1.03-2.01, p=0.032)
Extent of resection	partial res. or biopsy	112 (35.6)	-	-
	GTR or near GTR	203 (64.4)	0.38 (0.28-0.52, p<0.001)	0.36 (0.26-0.49, p<0.001)
Methylation subgroup	MES	158 (32.1)	-	-
	RTK I	120 (24.4)	1.40 (1.05-1.88, p=0.023)	1.19 (0.79-1.79, p=0.397)
	RTK II	214 (43.5)	1.29 (0.99-1.68, p=0.062)	1.32 (0.91-1.90, p=0.139)

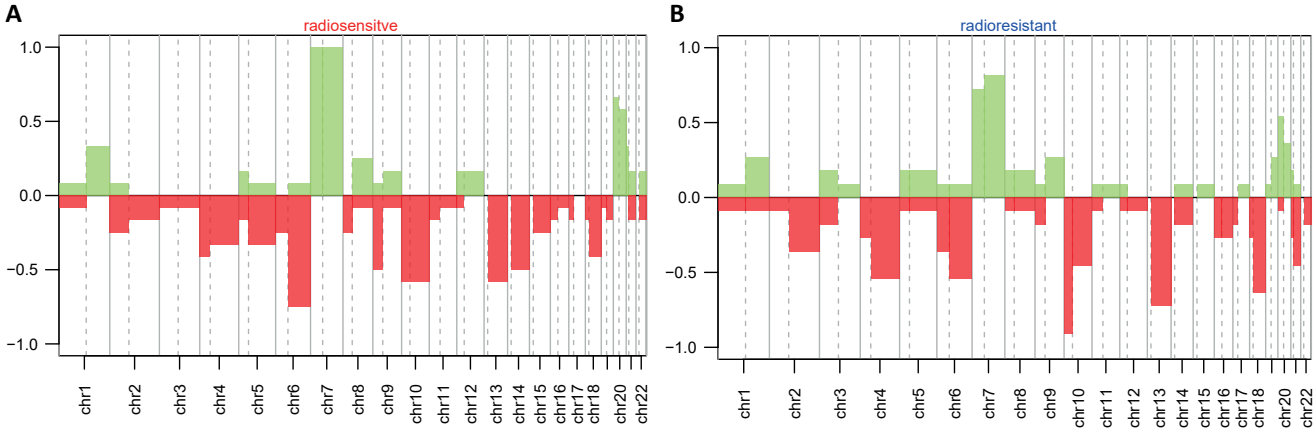
Supplementary Figure 3: Analysis of mean methylation within GBM methylation subgroups. Comparison of **A** Kaplan-Meier curves and **B** mean methylation of three subgroups. Kaplan-Meier curves for **C** MES, **D** RTK I, **E** RTK II dividing the subgroup cohorts at the respective best cut-off of mean methylation. **F** Multivariate analysis by including the methylation subgroup.



Supplementary Figure 4: Comparison of global mean methylation of all tumor and control methylation classes from Capper et al. (red: “GBM, MES”, “GBM, RTK I”, “GBM, RTK II”).[12]



Supplementary Figure 5: Analysis of different cell populations regarding the mean methylation, with the cohort divided at the best cut-off **A** myeloid, **B** monocyte, **C** glia, **D** neuron. **E** Proportion of myeloid cells in the three methylation subgroups.



Supplementary Figure 6: Cumulative copy number plots of **A** radiosensitive and **B** radioresistant cell lines.

4. Publikation 2

Der Inhalt dieser Arbeit wurde veröffentlicht in:

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DNA methylation subclasses predict the benefit from gross total tumor resection in IDH-wildtype glioblastoma patients

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Abstract

Background. DNA methylation-based tumor classification allows an enhanced distinction into subgroups of glioblastoma. However, the clinical benefit of DNA methylation-based stratification of glioblastomas remains inconclusive.

Methods. Multicentric cohort study including 430 patients with newly diagnosed glioblastoma subjected to global DNA methylation profiling. Outcome measures included overall survival (OS), progression-free survival (PFS), prognostic relevance of EOR and *MGMT* promoter methylation status as well as a surgical benefit for recurrent glioblastoma.

Results. 345 patients (80.2%) fulfilled the inclusion criteria and 305 patients received combined adjuvant therapy. DNA methylation subclasses *RTK I*, *RTK II*, and mesenchymal (MES) revealed no significant survival differences (RTK I: Ref.; RTK II: HR 0.9 [95% CI, 0.64–1.28]; $p = 0.56$; MES: 0.69 [0.47–1.02]; $p = 0.06$). Patients with *RTK I* (GTR/near GTR: Ref.; PR: HR 2.87 [95% CI, 1.36–6.08]; $p < 0.01$) or *RTK II* (GTR/near GTR: Ref.; PR: HR 5.09 [95% CI, 2.80–9.26]; $p < 0.01$) tumors who underwent gross-total resection (GTR) or near GTR had a longer OS and PFS than partially resected patients. The MES subclass showed no survival benefit for a maximized EOR (GTR/near GTR: Ref.; PR: HR 1.45 [95% CI, 0.68–3.09]; $p = 0.33$). Therapy response predictive value of MGMT promoter methylation was evident for *RTK I* (HR 0.37 [95% CI, 0.19–0.71]; $p < 0.01$) and *RTK II* (HR 0.56 [95% CI, 0.34–0.91]; $p = 0.02$) but not the MES subclass (HR 0.52 [95% CI, 0.27–1.02]; $p = 0.06$). For local recurrence (n = 112), re-resection conveyed a progression-to-overall survival (POS) benefit ($p < 0.01$), which was evident in *RTK I* ($p = 0.03$) and *RTK II* ($p < 0.01$) tumors, but not in MES tumors ($p = 0.33$).

Conclusion. We demonstrate a survival benefit from maximized EOR for newly diagnosed and recurrent glioblastomas of the *RTK I* and *RTK II* but not the MES subclass. Hence, it needs to be debated whether the MES subclass should be treated with maximal surgical resection, especially when located in eloquent areas and at time of recurrence.

Key Points

- The DNA methylation subclasses *RTK I, RTK II* and MES have a comparable OS and PFS after surgery and standard adjuvant therapy.
- A survival benefit of a maximized EOR exists in the *RTK I* and *RTK II* subclasses, but not the MES subclass.
- A methylated *MGMT* promoter is an independent prognostic factor for the OS in *RTK I* and *RTK II* tumors.
- For recurrent glioblastoma, a re-resection is associated with a prolonged POS in *RTK I* and *RTK II* tumors.

Importance of the Study

Maximal and safe resection is the cornerstone of glioblastoma therapy and extent of resection remains one of the main factors determining a more favorable prognosis. Hence, it is assumed that every glioblastoma patient will benefit from maximal resection. In the past years, global DNA methylation profiling has gained increased importance and allows a more precise distinction of IDH-wildtype glioblastoma into subclasses. However, thus far no real translation to the clinical practice from these extensive subclassification has been achieved. Our study investigated the utility of global methylation profiling to provide a methylation-based therapy guidance for glioblastoma patients, especially to predict the benefit of surgical resection. We report about a comparable overall survival between the DNA methylation subclasses *RTK* I, *RTK* II, and mesenchymal, but demonstrate that the known survival benefit of maximized extent of resection applies to *RTK* I and *RTK* II tumors but not to the MES subclass.

Maximal and safe resection is the cornerstone of glioblastoma therapy. Although tumors have usually already infiltrated far into the surrounding tissue, resulting inevitably in local and discontinuous recurrences, extent of resection (EOR) remains one of the main factors determining a more favorable prognosis.[1–3] Recent studies provided evidence that maximized EOR benefits the survival outcome regardless of the *O6-methylguanine DNA-methyltransferase (MGMT)* promoter methylation status.[3] Hence, it is assumed that every glioblastoma patient will benefit from maximal resection if feasible. Surgery is routinely followed by combined radiotherapy and chemotherapy with the DNA-alkylating agent temozolomide,[4] for which the *MGMT* promoter methylation status is a known predictive marker.[5–7]

In the past years, global DNA methylation profiling—the use of arrays to determine DNA methylation patterns across the genome—has been developed into a tool that increases the accuracy of exact molecular classification of central nervous system tumors with the potential to further stratify patients, beyond the assessment of routine clinicopathological characteristics.[8,9] Using the DNA methylation-based classifier, glioblastomas can be assigned to different subclasses, including receptor tyrosine kinase (*RTK*) *I*, *RTK II*, *RTK III*, H3.3 G34-mutant, midline, *MYCN* and mesenchymal (MES).[8] In adult patients, the most common methylation subclasses comprise *RTK I*, RTK II, and MES. For patient cohorts, which were mainly selected for clinical trials, the survival outcome was comparable between these three subclasses[10–12] and it was indicated that the prognostic benefit of a methylated *MGMT* promoter may be limited to the *RTK II* subclass in patients treated with monotherapy.[10] However, further prognostic significances of these epigenetic subgroups for the treatment of glioblastoma patients are largely unknown.

Here, in a multicenter cohort of 430 IDH-wildtype glioblastomas, we show that patients with MES tumors gain no significant advantage in overall survival (OS) or progression-free survival (PFS) from a gross total or near gross total resection. In contrast, OS and PFS are significantly prolonged by gross-total resection or near-gross total resection in patients with *RTK I* and *RTK II* tumors. We further show that a re-resection is only beneficial in *RTK I* and *RTK II* subclasses. Thus, by DNA methylation classification, we provide the basis for molecular stratification of glioblastoma patients who will or will not benefit from maximal resection at diagnosis or tumor recurrence. Our findings raise the question whether maximal resection of MES glioblastomas should be aimed for, especially when there is a risk of postoperative deficit.

Materials and Methods

Study Population

Glioma tissue from 430 patients who were newly diagnosed with IDH-wildtype glioblastoma, and who underwent

surgery at University Medical Center Hamburg-Eppendorf, University Hospital Frankfurt, or Charité University Hospital Berlin (all Germany) was analyzed. Patients underwent surgery between January 2012 and December 2021. Informed written consent was obtained from all patients. Diagnosis was based on the WHO classification.[13] The EOR was stratified into gross total resection (GTR), near GTR, and partial resection (PR) or stereotactic biopsy. A GTR was defined as a complete removal of contrast-enhancing parts, a near GTR as a removal of more than 90% of the contrast-enhancing parts, whereas a resection of lower than 90% was defined as PR/biopsy. The EOR of contrast-enhancing parts was evaluated by MRI performed up to 48 h after surgery. OS was calculated from diagnosis until death or last follow-up, and PFS from diagnosis until progression according to Response Assessment in Neuro-Oncology (RANO) criteria based on local assessment.[14] This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline.[15]

Inclusion Criteria

Inclusion criteria were defined based on DNA methylation profiling results and clinical data as presented in Figure 1. Methylation profiling results were submitted to the molecular neuropathology (MNP) methylation classifier v11b4 hosted by the German Cancer Research Center (DKFZ).[8] Patients were included if the calibrated score for methylation class family glioblastoma, IDH-wildtype was >0.84.[16] In addition, patients with a score below 0.84 but above 0.7 with a combined gain of chromosome 7 and loss of chromosome 10 or amplification of epidermal growth factor receptor (*EGFR*) were included in accordance with cIMPACT criteria.[17] Furthermore, to clearly separate the subclasses *RTK I*, *RTK II*, and MES, a class member score of ≥0.5 for one of these three subclasses was required. Patients had to further meet the following clinical criteria: supratentorial tumor localization, adjuvant treatment after surgery, age above 18 years, and availability of data for OS and PFS.

DNA Methylation Profiling

DNA was extracted from tumors and analyzed for genome-wide DNA methylation patterns using the Illumina EPIC (850k) array. Processing of DNA methylation data was performed with custom approaches as previously described.[16] Classification was performed using the MNP brain tumor classifier v11b4 of the DKFZ.[8,16] Evaluation of the *MGMT* promoter methylation status was made from the classifier output v11b4 using the STP27-method.

3D Volumetric Segmentation

We analyzed T1-weighted as well as T2-weighted FLAIR (Fluid attenuated inversion recovery) magnetic resonance imaging (MRI) axial images before surgery. The program BRAINLAB was used. The region of interest was delineated in every slice, enabling a multiplanar 3D reconstruction. The volume of contrast enhancement and FLAIR hyperintensity was assessed in cm^3.

Statistical Analysis

Differences in continuous variables were analyzed with the Mann–Whitney *U* test and differences in proportions were analyzed with the chi-square-test or Fisher exact test. Variables with possible prognostic effect were assessed by log-rank test and all survival curves were visualized as results from the Kaplan Meier analysis. Overall and progression-free survival, hazard ratios (HRs), and 95% confidence interval (CI) were computed for each group Cox proportional hazards regression model. The potential prognostic variables were age (continuous), gender (male, female), tumor location (frontal, parietal, temporal, occipital), hemisphere (left, right, both), number of lobe involvement (one, > one), extent of resection (GTR, near GTR, partial resection/biopsy), *MGMT* promoter status (methylated, nonmethylated), Karnofsky Performance Score (continuous), volume of contrast-enhancing tumor (continuous) and FLAIR lesion (continuous), and DNA methylation subclass (*RTK I*, *RTK II*, MES). All variables associated with OS or PFS with a *p*-value less than 0.05 in univariate analysis were included in the multivariable model. Multicollinearity was established between risk factors using correlation coefficient and Variance Inflation Factors (VIF). To avoid multicollinearity, a correlation coefficient >0.8 between any two variables was set as the threshold for multicollinearity. In addition, the threshold for VIF measured on the set of variables was set ≥ 4 to indicate the presence of multicollinearity. In the analyses of this study, calculated parameters did not reach these thresholds. In general, a two-sided *p*-value less than 0.05 was considered statistically significant. All analyses were performed using SPSS Inc. (Chicago, IL, USA). Data illustrations were performed using GraphPad Prism 9.

Results

Study Population

A total of 430 patients who were newly diagnosed with a glioblastoma were enrolled in this study. After applying the aforementioned inclusion criteria, 345 patients were available for further analysis. The mean age of the study population was 61.4 years. 122 patients (40.0%) were female. GTR was achieved in 38.4% and near GTR in 26.6% of patients, whereas 35.0% underwent partial resection or biopsy (Supplemental Table 1). In 153 patients (50.2%), a methylated *MGMT* promoter was diagnosed. The majority (88.4%) received radiochemotherapy as adjuvant treatment after surgery.

Clinical Data of DNA Methylation Subclasses

After DNA methylation profiling, patients were stratified according to their methylation subclass *RTK I* (31.1%), *RTK II* (41.0%) and MES (27.9%) (Figure 1A, Supplemental Table 1). Basic clinical characteristics, such as location, contrast enhancement volume, FLAIR volume and EOR did not differ between the subclasses (Supplemental Table 1). Patients with *RTK I* tumors were significantly older than patients with

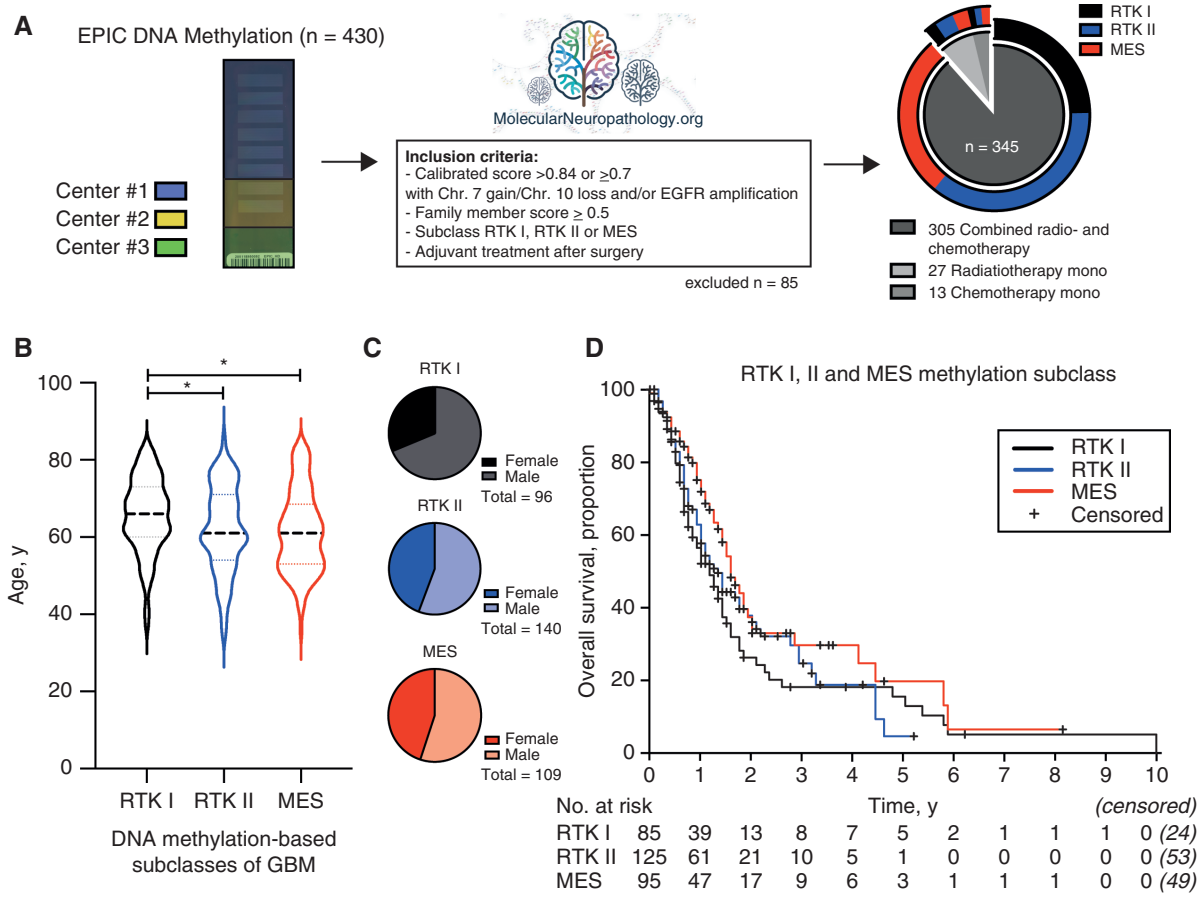

Fig. 1 Overview of the study representing the (A) inclusion criteria and included patients stratified according to their adjuvant therapeutic regimen, (B) age, and (C) methylation subclasses. (D) Overall survival of all patients in accordance with the respective methylation subclasses. For the *RTK I* group, median overall survival was 14.0 (95% CI, 9.9–18.2) months (multivariable hazard ratio [HR], 1 [reference]); *RTK II*, 16.0 (95% CI, 12.3–19.7) months (multivariable HR, 0.56 [95% CI, 0.64–1.28]; $p = 0.56$); MES, 19.0 (95% CI, 15.4–22.6) months (multivariable HR, 0.69 [95% CI, 0.47–1.02]; $p = 0.06$).

RTK II or MES tumors (Figure 1B, Supplemental Table 1), without a significant difference in sex distribution (Figure 1C). Karnofsky performance status after diagnosis ($p = 0.64$) and *MGMT* promoter methylation status ($p = 0.83$) were comparable in all three groups (SupplementalTable 1).

Impact of EOR in DNA Methylation Subclasses

305 patients (88.4%) received radiochemotherapy after surgery and were included for further survival analysis (Figures 1–3). At a mean (SD) follow-up time of 14.5 (15.3) months, 179 deaths (58.6%) were observed. During the study period, 170 patients (55.7%) showed tumor progression.The median length of survival for all patients from the time of index surgery was 15.8 months (range 1–97 months). After stratifying the study population which were treated with combined therapy according to their methylation subclass, survival analyses revealed no significant differences for OS ($p = 0.06$, Figure 1D) and PFS ($p = 0.52$, Supplemental Figure 1A) between these groups. Patients who received a GTR (Figure 2A and 2B) had a significantly longer OS (HR, 2.18; 95% CI, 1.46–3.24; $p < 0.01$) (Figure 2C) and PFS (HR, 1.78; 95% CI, 1.19–2·65; $p < 0.01$) (Supplemental Figure 1B) using Cox proportional hazards regression model (Supplemental Tables 2 and 3). When examining the impact of maximal EOR in each methylation subclass a significant prolongation of OS in *RTK I* (HR, 2.87; 95% CI, 1.36–6.08; $p < 0.01$) (Figure 2D) and *RTK II* tumors was found (HR, 5.09; 95% CI, 2.80–9.26; $p < 0.01$) (Figure 2E). However, a prolonged OS for a GTR or near GTR was not observed in the MES subclass (HR, 1.45; 95% CI, 0.69–3.09; $p = 0.33$) (Figure 2F). A significantly longer PFS of a GTR or near GTR was seen for *RTK II* tumors, but not the *RTK I* and MES subclasses (Supplemental Figures 1C-E). Detailed information for univariate and multivariate analysis for each subclass are available for OS in SupplementalTables 4–6 and for PFS in SupplementalTables 7–9.

Impact of *MGMT* Promoter Methylation Status in DNA Methylation Subclasses

Of 305 combined-treated patients, 153 (50.2%) had a methylated *MGMT* promoter status, and DNA methylation

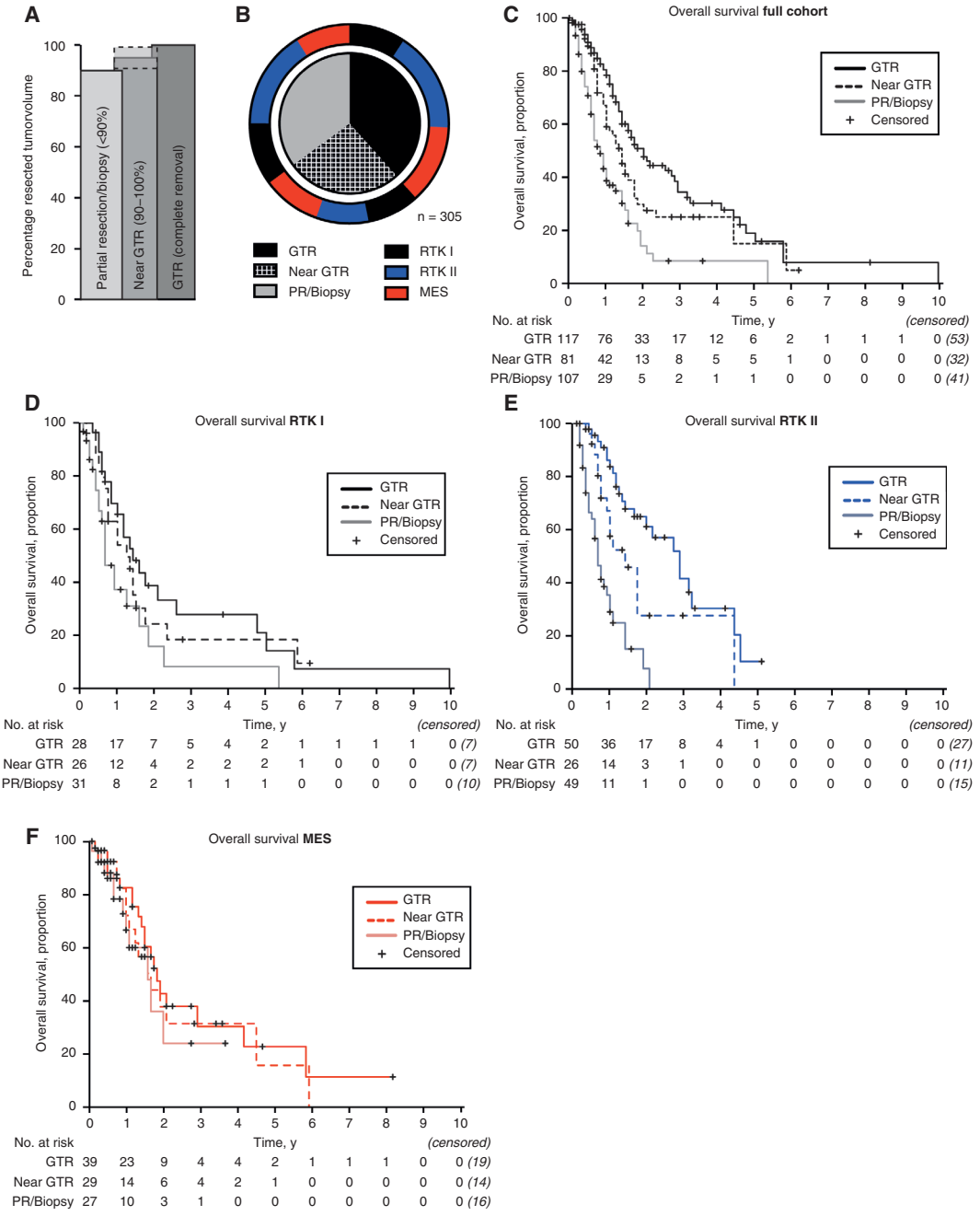

Fig. 2 Visualization of patients receiving combined radiochemotherapy after surgery. The study population was further stratified to the (A) extent of resection, and (B) methylation subclasses to analyze the overall survival (OS). In all survival analyses patients were stratified into the following groups: gross total resection (GTR), near GTR, and partial resection (PR) or biopsy. (C) includes all methylation subclasses. For the GTR group, median overall survival was 24.0 (95% CI, 17.9–30.1) months (multivariable hazard ratio [HR], 1 [reference]); near GTR, 17.0 (95% CI, 13.6–20.4) months (multivariable HR, 0.97 [95% CI, 0.64–1.46]; $p = 0.88$); PR/biopsy, 10.0 (95% CI, 7.9–12.1) months (multivariable HR, 2.18 [95% CI, 1.46–3.24]; $p < 0.01$). (D) includes *RTK I* tumors. For the GTR group, median overall survival was 17.0 (95% CI, 9.6–24.4) months (multivariable hazard ratio [HR], 1 [reference]); near GTR, 15.0 (95% CI, 7.1–22.9) months (multivariable HR, 0.93 [95% CI, 0.44–1.95]; $p = 0.85$); PR/biopsy, 8.0 (95% CI, 4.9–11.0) months (multivariable HR, 2.87 [95% CI, 1.36–6.08]; $p < 0.01$). (E) includes *RTK II* tumors. For the GTR group, median overall survival was 35.0 (95% CI, 23.8–46.2) months (multivariable hazard ratio [HR], 1 [reference]); near GTR, 17.0 (95% CI, 10.5–23.6) months (multivariable HR, 1.63 [95% CI, 0.83–3.21]; $p = 0.16$); PR/biopsy, 8.0 (95% CI, 6.0–9.9) months (multivariable HR, 5.09 [95% CI, 2.80–9.26]; $p < 0.01$). (F) includes MES tumors. For the GTR group, median overall survival was 21.0 (95% CI, 16.8–25.2) months (multivariable hazard ratio [HR], 1 [reference]); near GTR, 19.0 (95% CI, 11.7–26.3) months (multivariable HR, 1.15 [95% CI, 0.59–3.25]; $p = 0.68$); PR/biopsy, 18.0 (95% CI, 9.9–26.1) months (multivariable HR, 1.45 [95% CI, 0.68–3.09]; $p = 0.33$).

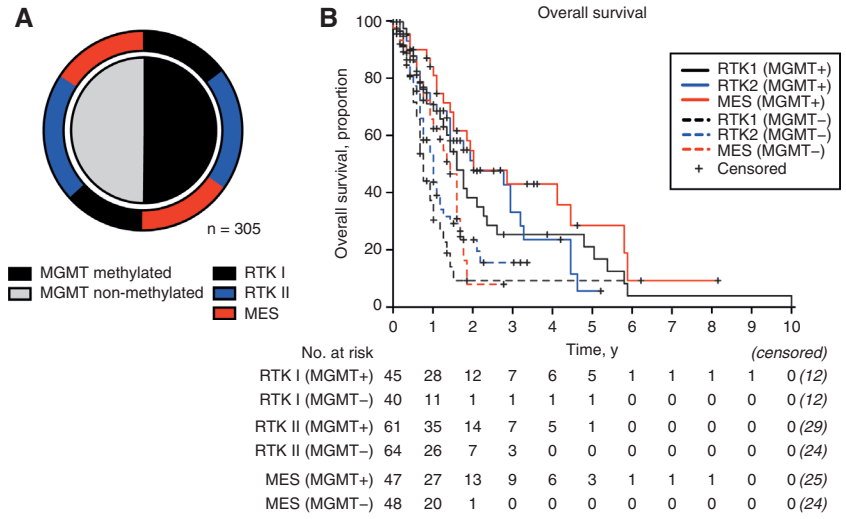

Fig. 3 ***MGMT* promoter status in the methylation subclasses of patients receiving combined radiochemotherapy.** (A) 153/305 patients (50.2%) had a methylated *MGMT* promoter status with 45 (52.3%) being methylated in the *RTK I*, 61 (48.8%) in the *RTK II*, and 47 (49.5%) in the MES subclass. (B) Patients were stratified in accordance with their methylation subclass and *MGMT* promoter status. In *RTK I* tumors, the nonmethylated *MGMT* group had a median overall survival of 9.0 (95% CI, 7.4–10.6) months (multivariable hazard ratio [HR], 1 [reference]); methylated *MGMT* group, 19.0 (95% CI, 14.6–23.4) months (multivariable HR, 0.37 [95% CI, 0.19–0.71]; $p < 0.01$). In *RTK II* tumors, the nonmethylated *MGMT* group had a median overall survival of 12.0 (95% CI, 9.2–14.8) months (multivariable hazard ratio [HR], 1 [reference]); methylated *MGMT* group, 24.0 (95% CI, 8.3–39.7) months (multivariable HR, 0.56 [95% CI, 0.34–0.91]; $p = 0.02$). In MES tumors, the nonmethylated *MGMT* group had a median overall survival of 17.0 (95% CI, 13.9–20.0) months (multivariable hazard ratio [HR], 1 [reference]); methylated *MGMT* group, 24.0 (95% CI, 7.5–40.5) months (multivariable HR, 0.52 [95% CI, 0.27–1.02]; $p = 0.06$).

subgroups were equally distributed among methylated and nonmethylated tumors (Figure 3A). As expected, a methylated *MGMT* promoter status was predictive of favorable OS (HR, 0.51; 95% CI, 0.37–0.72; $p < 0.01$) (Supplemental Table 2) and PFS (HR, 0.44; 95% CI, 0.31–0.62; $p < 0.01$) (Supplemental Table 3) in the combined cohort. These results were also observed for the individual DNA methylation subclasses by univariate analysis (Supplemental Tables 4–9). However, after adjusting for covariates using Cox regression model, the *MGMT* promoter methylation status remained an independent factor for a favorable outcome in *RTK I* (HR, 0.37; 95% CI, 0.19–0.71; $p < 0.01$) and *RTK II* (HR, 0.56; 95% CI, 0.34–0.91; $p = 0.02$) tumors, but not the MES subclass (HR, 0.52; 95% CI, 0.27–1.02; $p = 0.06$) (Figure 3B). Similar results were observed for PFS (Supplemental Figure 2, Supplemental Tables 7–9).

Outcome after Local Recurrence in DNA Methylation Subclass

We identified 68 patients who were operated for local recurrent glioblastoma. Clinical characteristics are detailed in Supplemental Table 10. To investigate the benefit of a recurrence resection as a therapeutic option prior to second line therapy, we compared these 68 patients who underwent a re-resection and received adjuvant therapy with 44 patients without a re-resection before starting second line therapy (Figure 4A). Both groups included patients with local recurrence and previously completed combined radiochemotherapy. Survival analysis revealed a favorable progression-to-overall survival (POS) time for the re-resection cohort (HR, 2.56; 95% CI, 1.34–5.01; $p < 0.01$) (Figure 4B). Here, it must be taken into consideration that patients who underwent re-resection were more likely to receive combined therapy as second line adjuvant therapy ($p < 0.01$, Supplemental Table 10). After stratifying according to the respective methylation subclasses obtained from the primary tumor, the survival benefit was valid for patients with *RTK I* (HR, 2.82; 95% CI, 1.12–7.08; $p = 0.03$) (Figure 4C) and *RTK II* tumors (HR, 3.47; 95% CI, 1.55–7.78; $p < 0.01$) (Figure 4D), but not MES subclass (HR, 1.62; 95% CI, 0.61–4.29; $p = 0.33$) (Figure 4E).

Heterogeneity of DNA Methylation Subclasses

Since former studies reported on intratumoral DNA methylation heterogeneity within glioblastomas,[18,19] we investigated whether the methylation subclasses were stable during the course of disease progression. We therefore performed global DNA methylation analysis on 22 matched samples obtained from surgery at initial diagnosis and surgery at first recurrence. In 21 patients with a valid classifier output, we observed a switch of the DNA methylation subclass in 5 (23.8%) cases (Figure 4F). A change of the *MGMT* promoter methylation status occurred in 1 (4.8%) case.

Outcome of Patients ≥ 70 Years of Age in DNA Methylation Subclasses

From the main cohort treated with radiochemotherapy after surgery, we identified 70 patients who were 70 years of age

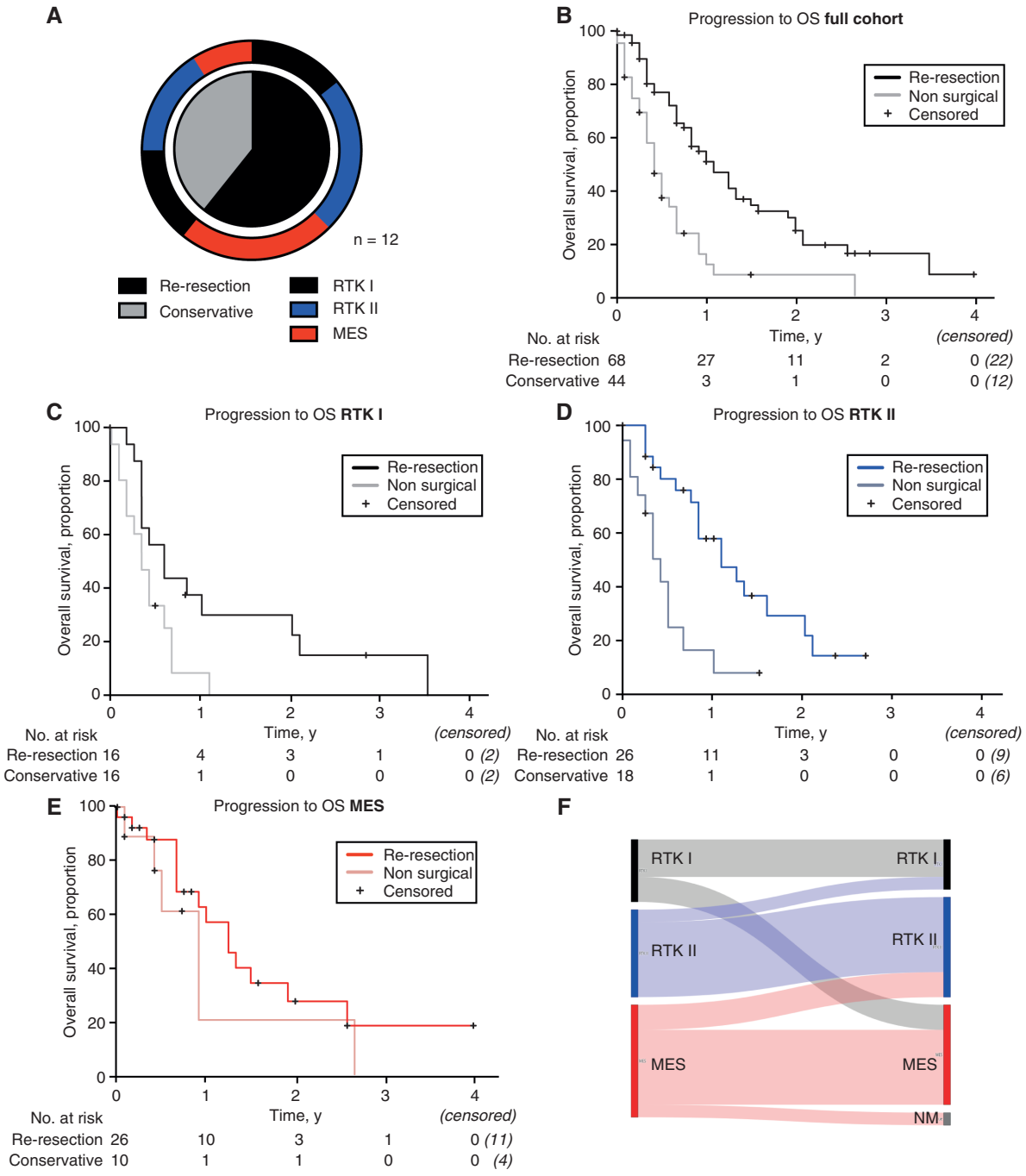

Fig. 4 Comparison of patients with a local recurrence receiving second line therapy with or without recurrence surgery. (A) 68 patients who underwent recurrence surgery were matched to 44 patients receiving second line therapy with comparable proportions of methylation subclasses in both cohorts. For the survival analyses, patients were stratified into a re-resection and nonsurgical group. (B) includes all patients. For the re-resection group, median progression-to-overall survival (POS) was 13.0 (95% CI, 8.9–17.1) months (multivariable hazard ratio [HR], 1 [reference]); non-surgical group, 5.0 (95% CI, 3.4–6.6) months (multivariable HR, 2.18 [95% CI, 1.46–3.24]; $p < 0.01$). (C) includes *RTK I* patients. For the re-resection group, median POS was 7.0 (95% CI, 3.1–10.9) months (multivariable hazard ratio [HR], 1 [reference]); nonsurgical group, 4.0 (95% CI, 2.1–5.9) months (multivariable HR, 2.82 [95% CI, 1.12–7.08]; $p = 0.03$). (D) includes *RTK II* patients. For the re-resection group, median POS was 13.0 (95% CI, 6.4–19.6) months (multivariable hazard ratio [HR], 1 [reference]); non-surgical group, 5.0 (95% CI, 2.9–7.1) months (multivariable HR, 3.47 [95% CI, 1.55–7.78]; $p < 0.01$). (E) includes MES patients. For the re-resection group, median POS was 15.0 (95% CI, 9.7–20.3) months (multivariable hazard ratio [HR], 1 [reference]); nonsurgical group, 11.0 (95% CI, 6.7–15.3) months (multivariable HR, 1.62 [95% CI, 0.61–4.29]; $p = 0.33$). (F) Sankey plot comparing the methylation subclasses of 22 matched pair samples between first and second surgery. A switch of the subclass was observed in 5 (20.0%) patients. *NM=no match.*

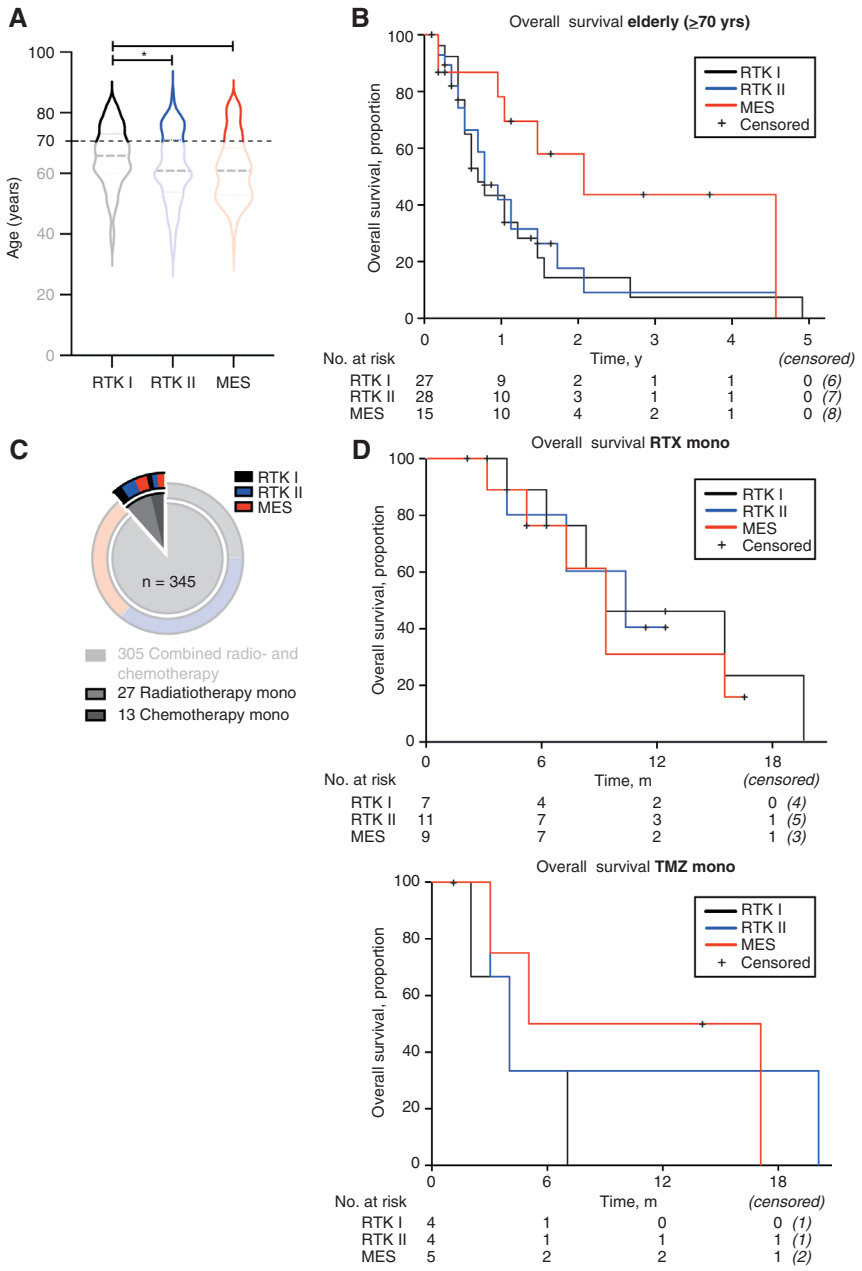

Fig. 5 Further analyses of elderly patients receiving combined therapy and all patients treated with monotherapy. For survival analyses, patients were stratified according to their methylation subclasses. (B) included all patients above 70 years of age with a Karnofsky Performance Score of at least 60. For the *RTK I* group, median overall survival was 8.0 (95% CI, 5.3–10.8) months (multivariable hazard ratio [HR], 1 [reference]); *RTK II*, 9.0 (95% CI, 5.6–12.4) months (multivariable HR, 0.62 [95% CI, 0.44–1.63]; $p = 0.62$); MES, 24.0 (95% CI, 7.9–40.0) months (multivariable HR, 0.35 [95% CI, 0.13–0.9]; $p = 0.03$). (D) included all patients who received radiation for monotherapy after surgery. For the *RTK I* group, median overall survival was 10.0 (95% CI, 3.6–16.4) months (multivariable hazard ratio [HR], 1 [reference]); *RTK II*, 9.0 (95% CI, 2.2–15.8) months (multivariable HR, 0.94 [95% CI, 0.22–4.04]; $p = 0.93$); MES, 9.0 (95% CI, 6.7–11.3) months (multivariable HR, 1.24 [95% CI, 0.29–5.13]; $p = 0.77$). (E) included all patients who received temozolomide for monotherapy after surgery. For the *RTK I* group, median overall survival was 4.0 (95% CI, 0.8–7.2) months (multivariable hazard ratio [HR], 1 [reference]); *RTK II*, 4.0 (95% CI, 2.4–5.6) months (multivariable HR, 0.44 [95% CI, 0.07–2.89]; $p = 0.39$); MES, 5.0 (95% CI, 0.0–14.2) months (multivariable HR, 0.45 [95% CI, 0.09–2.34]; $p = 0.34$).

and older, and who had a Karnofsky performance score of at least 60% (Figure 5A). Survival analyses revealed a significantly longer OS for the MES subclass (HR, 0.35; 95% CI, 0.13–0.90; $p = 0.03$) (Figure 5B). In addition, the EOR (HR, 2.39; 95% CI, 1.07–5.34; $p = 0.03$) and Karnofsky performance score (HR, 0.96; 95% CI, 0.94–0.99; $p < 0.01$) were independent prognostic factors in this distinctive patient cohort (Supplemental Table 11).

Outcome after Monotherapy in DNA Methylation Subclasses

Of all 345 patients, 40 patients (10.3%) were treated with monotherapy after surgery, of whom 27 (67.5%) received radiation and 13 (32.5%) TMZ (Figure 5C). After stratifying these patients according to their methylation subclasses, basic clinical characteristics were balanced between these groups (Supplemental Table 12). Within the monotherapeutically treated patients, the methylation subclass was not associated with OS, neither for treatment with TMZ (HR, 0.45; 95% CI, 0.09–2.34; $p = 0.34$) (Figure 5D top) nor radiation (HR, 1.24; 95% CI, 0.29–5.13; $p = 0.77$) (Figure 5D bottom) (Supplemental Tables 13–15).

Discussion

In recent years, DNA methylation-based tumor profiling facilitates a more precise classification and distinction of brain tumor subgroups. For glioblastoma, *RTK I*, *II,* and MES are the most common subclasses. *RTK I* tumors are enriched for *platelet-derived growth factor receptor A (PDGFRA)* gene amplification, whereas *RTK II* tumors frequently harbor amplification of *epidermal growth factor receptor* (*EGFR*) gene, while MES tumors have no typical recurrent mutations.[12,20,21] Although an overlap was seen between the methylation-based subclassification and transcriptome analysis,[22] DNA methylation does not completely match with RNA-based subclasses or the proteogenomic and metabolomic landscape of glioblastomas.[21] Nevertheless, thus far no real translation to the clinical practice from these extensive subclassification has been achieved. Our study investigated the utility of global methylation profiling (850K) to provide a methylation-based therapy guidance for glioblastoma patients.

We present the following major findings: (1) The DNA methylation subclasses *RTK I*, *RTK II* and MES have a comparable OS and PFS after surgery and standard adjuvant therapy. (2) A survival benefit of a maximized EOR exists in the *RTK I* and *RTK II* subclasses, but not the MES subclass. (3) A methylated *MGMT* promoter is an independent prognostic factor for the OS in *RTK I* and *RTK II* tumors but did not reach statistical significance in the MES subclass even though a survival prolongation was observed. (4) For recurrent glioblastoma, a re-resection is associated with a prolonged POS in *RTK I* and *RTK II* tumors. (5) Elderly patients of 70 years of age and older who receive radiochemotherapy have a favorable OS and PFS in the MES subclass.

The Neurooncology Working Group (NOA)-8 and EORTC-26101 trials showed a comparable survival outcome between the *RTK I*, *RTK II,* and MES methylation subclasses,[10,11] which we also demonstrated in our study. Only MES tumors tended to have a longer OS, which may relate to a higher number of tumor-infiltrating lymphocytes,[12,23] resembling a more immunogenic tumor. However, the relationship of infiltrating lymphocytes and prognosis in glioblastoma remains controversial.[12,24–26]

Surgical resection remains the cornerstone in glioblastoma therapy. It is well established that the EOR represents an independent factor for OS and PFS, regardless of the IDH and *MGMT* promoter methylation status.[1,3,27] Therefore, the surgical treatment goal remains to achieve a GTR of the contrast-enhancing tumor parts without a postoperative neurological deterioration. However, DNA methylation profiles were never considered as a prognostic factor for the survival benefit of maximized resected tumors. Conducting 430 global methylation profiles we found that the survival benefit of gross-totally resected glioblastomas was limited to *RTK I* and *RTK II* methylation subclasses. In patients with tumors classified as MES subclass and treated with adjuvant radiochemotherapy, we found no significant difference in OS and PFS between biopsied, partially resected, or gross totally resected tumors. These results raise the question of whether patients with a MES glioblastoma should invariably undergo maximal resection when postoperative neurologic deterioration is likely to occur, thus preventing adjuvant therapy. Conversely, this also implies that achieving GTR in *RTK I* and *RTK II* tumors is of particular importance. However, it should be noted that DNA methylation profiling to determine the subclass postsurgery takes approximately 1 week. Therefore, rapid testing for DNA methylation subclasses is desired, preferably preoperative, or intraoperative in cases with a glioblastoma-suspected MRI scan. To this end, a promising approach was pursued by Djirackor and colleagues who achieved a high accuracy of intraoperative DNA methylation-based classification of CNS tumors using Nanopore ultra-low coverage whole genome sequencing and obtained results within 120 min in most cases.[28,29] In this rapidly evolving field, additional approaches are currently being presented that raise the hope of intraoperatively determination of DNA methylation subclasses.[30,31]

Besides the well-defined prognostic impact of EOR, the importance of the *MGMT* promoter methylation status is undeniable, since favorable survival has been historically demonstrated in patients with a methylated *MGMT* promoter.[32–34] In our cohort who underwent combined treatment after surgery, a prolonged survival of *MGMT*-methylated tumors was seen in all patients, especially in the *RTK I* and *RTK II* subclass, while there was no difference in the proportion of a methylated *MGMT* promoter between the subclasses. For MES tumors, it needs to be addressed that the survival outcome of *MGMT*-methylated tumors was more favorable than tumors with a nonmethylated *MGMT* promoter, even though a statistical significance was not reached. Therefore, choosing adjuvant therapy dependent on the *MGMT* promoter methylation status seems to be justified for MES tumors. Since it remains difficult to find the optimal therapeutic regimen for patients of older age, we defined a subgroup of septuagenarians (70 years and older and Karnofsky score of at least 60%) treated with radiochemotherapy. In this subgroup, the MES subclass was associated with a favorable survival. These results underscore the previously mentioned findings that treatment response, whether monotherapy or radiochemotherapy, and the benefit of a methylated *MGMT* promoter depend on DNA methylation subclasses as well as patient characteristics.

Despite numerous efforts, there is still no standard of care for recurrent glioblastoma, and the decision to re-operate depends on the general condition of the

patient and the local or multifocal recurrence.[35] In our study, we compared a group of patients with a local recurrence of the same age, Karnofsky index and *MGMT* promoter methylation status who underwent re-resection and adjuvant therapy or conservative treatment alone. In the *RTK I* and *RTK II* subclasses, but not in the MES subclass, longer survival was observed between the aforementioned groups. Thus, the decision to re-operate at the time of recurrence might have a more favorable survival advantage in *RTK I* and *RTK II* than in MES glioblastomas. Because determining the methylation subclass at the time of initial surgery is currently impractical, methylation-based decision making at the time of disease recurrence may be useful.

Although the methylation subclass was changed in 24.8% of tumors this epigenetic imprint appears more stable than transcriptional subtyping, in which 46.0% of tumors had an altered subtype.[36] Till date, it is largely unknown how switching DNA methylation subclasses can potentially affect the prognosis of glioblastoma patients, as the study of temporal heterogeneity has been limited to transcriptional subtypes. In our study, transition had no effect on patient outcome, and the MES subclass remains the most stable tumor subclass.

In summary, we show that patients with MES tumors gain no significant advantage in OS or PFS from GTR or near-GTR compared to patients with *RTK I* and *RTK II* tumors, which have a prolonged OS and PFS if a maximized resection is performed. We further demonstrate that a re-resection is only beneficial in *RTK I* and *RTK II* tumors for local recurrences. Thus, by methylation classification, we provide the basis for molecular stratification of glioblastoma patients who will or will not benefit from maximal resection at diagnosis or at tumor recurrence.

Limitations

This study has several limitations. The retrospective cohort includes patients from three major neuro-oncology centers rather than from a prospective randomized clinical trial. However, this would also be difficult to ethically justify to evaluate the benefit of maximal resection. Our cohort consisted of patients only amendable for biopsy and in which complete resection was anticipated but uncertain due to the conduct of surgery and the feedback obtained by intraoperative neuromonitoring, hence explaining the relatively low rate of gross-total resected tumors. An independent validation cohort is lacking, but a comparison across centers showed consistent results. No data for toxicity and adverse events during adjuvant therapy was available. Additionally, treatment decisions for surgical resections at time of local recurrence could be influenced by surgeon and patient perspectives, which is not documented in this study and the numbers within subgroups of patients that received monotherapy or were treated at relapse were small and statistical analysis may be of limited reliability. In the OS analysis between DNA methylation subgroups and the prognostic impact of MGMT promoter methylation status, we found a trend for the MES subclass in both categories, but this did not reach statistical significance. It is worth considering that this effect could also be achieved in a larger cohort or with a smaller number of censored data.

Conclusion

This study evaluated the prognostic relevance of DNA methylation profiling in a large, multi-institutional study population diagnosed with glioblastoma who received standard of care treatment and was followed until tumor recurrence and death. Although overall survival did not differ between DNA methylation subclasses, our results showed that the known survival benefit of maximized EOR applies to *RTK I* and *RTK II* tumors but not to the MES subclass. Therefore, it must be discussed whether the MES subclass should invariably be treated with maximal surgical resection, especially at tumor progression.

Supplementary Material

Supplementary material is available at *Neuro-Oncology* online.

Keywords

DNA methylation | extent of resection | gbm | glioblastoma | glioma | subgroup

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Conflict of interest statement. None declared.

Authorship statement. Dr Drexler and Dr Ricklefs had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: Drexler, Ricklefs, Schüller, Dührsen, Westphal. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: Drexler, Ricklefs, Dührsen, Lamszus, Schüller, Westphal. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: Drexler, Ricklefs, Eckhardt, Sauvigny. Obtained funding: Ricklefs, Westphal, Schüller. Administrative, technical, or material support: Eckhardt, Filipski, Siewert, Sauvigny. Supervision: Ricklefs, Dührsen, Schüller, Westphal.

Data availability

Individual patient-level data are available for the retrospective cohorts upon request.

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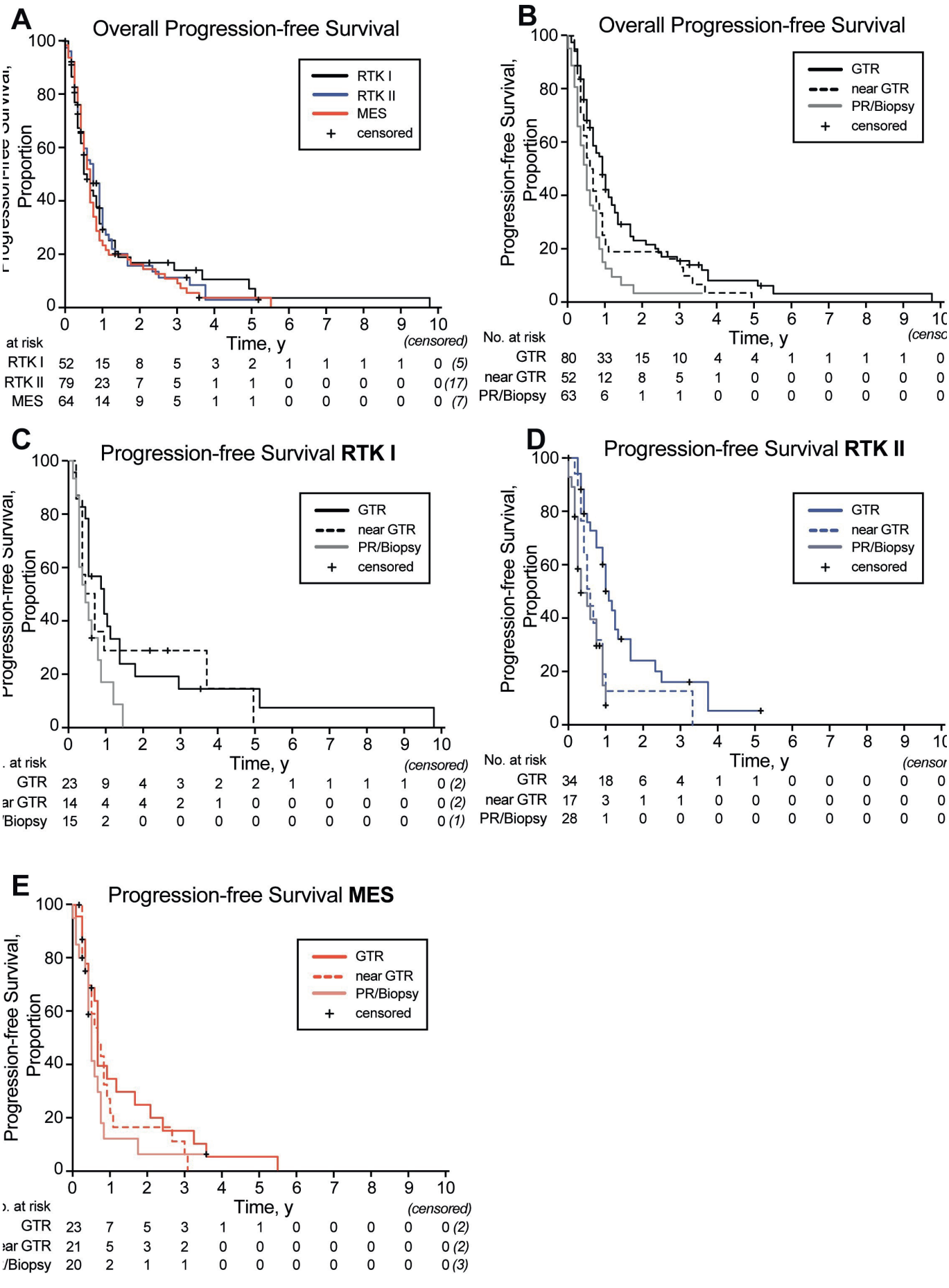
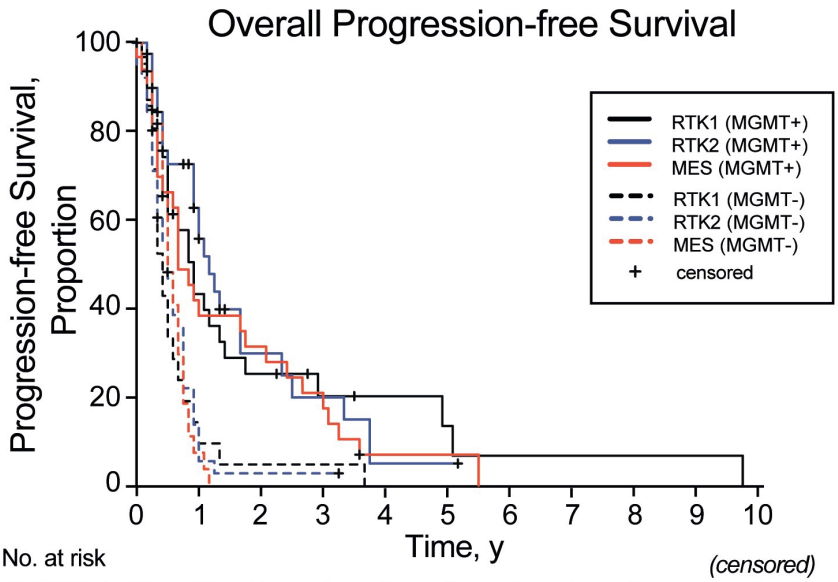


supp. Figure 1



No. at risk												(censored)
RTK I (MGMT+)	31	12	7	4	3	2	1	1	1	1	0	*(5)*
RTK I (MGMT-)	21	3	1	1	0	0	0	0	0	0	0	*(0)*
RTK II (MGMT+)	41	18	6	4	1	1	0	0	0	0	0	*(15)*
RTK II (MGMT-)	38	5	1	1	0	0	0	0	0	0	0	*(2)*
MES (MGMT+)	31	12	9	6	1	1	0	0	0	0	0	*(3)*
MES (MGMT-)	33	2	0	0	0	0	0	0	0	0	0	*(4)*

supp. Figure

5. Zusammenfassung

Die Überlebensprognose von Glioblastom-Patienten und -Patientinnen, die bereits die bestmögliche Therapie erhalten, ist mit 15 Monaten im Median nach Diagnose sehr kurz. Sowohl personalisierte Therapiemöglichkeiten als auch gefundene Biomarker sind unzureichend, um Patienten und Patientinnen ausschließlich mit effektiven Therapien zu behandeln.

Die epigenetischen Cluster-Analysen wurden anhand von 23 Zelllinien und 492 Tumorproben, die sowohl Chemo- als auch mit Strahlentherapie erfahren haben, angefertigt. Dabei wurden zunächst keine signifikanten Unterschiede zwischen verhältnismäßig lang überlebenden und kurz überlebenden Patienten und Patientinnen gefunden (Publikation 1 [14]).

Für die Kinom-spezifischen Muster wurden 21 Zelllinien und 11 Tumorproben verwendet. Es konnten auch hier keine therapierelevanten Cluster gefunden werden. Ein signifikanter Unterschied in der mittleren relativen Kinase-Aktivität zwischen strahlenresistenten und strahlensensitiven Zelllinien konnte ebenfalls nicht gefunden werden (siehe 1.3.2).

Zudem zeigte sich anhand klinischer Daten zu 305 Tumorproben, dass Patienten und Patientinnen der MES-Subgruppe keinen signifikanten Überlebensvorteil haben, wenn ihnen mehr als 90% des Tumors entfernt wurde, verglichen mit einem geringeren Resektionsausmaß. Ebenso ist für diese Subgruppe eine erneute Operation bei einem Rezidiv nicht vorteilhaft bezüglich des Überlebens. Diesen Patienten und Patientinnen könnte man eine erneute Operation und damit auch das Risiko auf Komplikationen ersparen (Publikation 2 [15]).

Die weitere Analyse der 492 Tumorproben ergab, dass die mittlere globale DNA-Methylierung einen neuen prognostischen Marker darstellte, der unabhängig von den bereits bekannten Markern (*MGMT*-Promotor-Methylierung, Alter, Resektionsausmaß) war. Bei Kombination dieser vier Marker lassen sich präzisere Vorhersagen auf das Überleben machen als mit den bekannten Markern, weil die mittlere Methylierung eine essentielle Rolle in der Risikogruppen-Stratifizierung spielte (Publikation 1 [14]). Außerdem konnte auch in 148 Patienten und Patientinnen, die zusätzlich zur Operation nur eine Strahlentherapie und keine Chemotherapie erhalten haben, ein signifikanter Unterschied im Überleben festgestellt werden. Patienten und Patientinnen, deren Tumorprobe eine höhere mittlere Methylierung aufweist, überleben länger als mit einer geringeren mittleren Methylierung, was auf eine erhöhte Strahlenempfindlichkeit hindeutet. Dies konnte in Zelllinien bestätigt werden. Im Falle von 23 Zelllinien, die nur bestrahlt wurden, konnte nämlich gezeigt werden, dass eine höhere mittlere Methylierung mit einer höheren Strahlensensitivität korreliert (Publikation 1 [14]).

Insgesamt wurden mit dieser Studie neue Erkenntnisse bezüglich des Therapieansprechens und der Überlebensprognose von Glioblastom-Patienten und -Patientinnen erzielt, die das

Potential haben, ein Baustein in der Therapieentwicklung zu sein, um langfristig die Therapie von Glioblastomen gezielter anzupassen und somit zu verbessern.

6. Abstract

The median overall survival of glioblastoma patients who are already receiving the best possible therapy is very short, with 15 months after diagnosis. Both personalized therapy options and identified biomarkers are insufficient to treat patients exclusively with effective therapies.

The epigenetic cluster analyses were based on 23 cell lines and 492 tumor samples that had received both chemotherapy and radiotherapy. Initially, no significant differences were found between relatively long-surviving and short-surviving patients (publication 1 [14]).

For the kinome-specific patterns, 21 cell lines and 11 tumor samples were used. No therapy-relevant clusters were found here either. A significant difference in the mean relative kinase activity between radioresistant and radiosensitive cell lines was also not found (see 1.3.2).

In addition, clinical data on 305 tumor samples showed that patients in the MES subgroup do not have a significant better overall survival if more than 90% of the tumor is resected compared to a smaller extent of resection. For this subgroup, a second resection in the event of a recurrence is also not advantageous in terms of overall survival. These patients could be spared a second surgery and thus also the risk of complications (publication 2 [15]).

Further analysis of the 492 tumor samples yielded that mean global DNA methylation was a new prognostic marker that was independent of the already known markers (MGMT promoter methylation, age, extent of resection). When these four markers are combined, more precise predictions of survival can be made than with the known markers, because the mean global DNA methylation played an essential role in risk group stratification. Furthermore, a significant difference in overall survival was also found in 148 patients who received only radiotherapy and no chemotherapy in addition to surgery. Patients whose tumor sample has a higher mean global methylation survive longer than those with a lower mean global methylation, which indicates an increased radiosensitivity. This was confirmed in cell lines. In the case of 23 cell lines that were only irradiated, it was shown that a higher mean global DNA methylation correlates with a higher radiosensitivity (publication 1 [14]).

Overall, this study provided new insights into the treatment response and survival prognosis of glioblastoma patients, which have the potential to be a component in therapy development in order to adapt and thus improve the treatment of glioblastomas in the future.

7. Erklärung des Eigenanteils an den Publikationen

Publikation 1:

Für diese Publikation wurde die Akquisition der Daten betrieben. Zudem erfolgte meinerseits die Analyse, inklusive der statistischen Auswertung, der bioinformatischen Analyse sowie Interpretation der Daten. Das Manuskript wurde von mir geschrieben und die dazugehörigen Abbildungen wurden ebenfalls von mir angefertigt. Abschließend erfolgte die Einarbeitung der Kommentare der Co-Autoren und Co-Autorinnen. In dem Revisions-Prozess habe ich die Reviewer-Kommentare bearbeitet und die weiteren geforderten Analysen dafür durchgeführt.

Publikation 2:

Für diese Publikation habe ich bei der Akquisition der Daten mitgearbeitet. Zudem unterstützte ich bei der Analyse und Interpretation der Daten, wobei ich größtenteils an der Analyse des Subgruppenwechsels zwischen der ersten und zweiten Resektion beteiligt war. Zudem unterstützte ich die Haupt-Autoren bei der statistischen Analyse und beteiligte mich an der Administration und dem technischen Support. Das Manuskript habe ich kritisch revidiert.

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9. Liste der Publikationen

Der Hauptteil dieser Arbeit wurde publiziert in:

1.)

Eckhardt A, Drexler R, Schoof M, Struve N, Capper D, Jelgersma C, Onken J, Harter PN, Weber KJ, Divé I, Rothkamm K, Hoffer K, Klumpp L, Ganser K, Petersen C, Ricklefs F, Kriegs M[+], Schüller U[+]. Mean global DNA methylation serves as independent prognostic marker in IDH-wildtype glioblastoma. *Neuro Oncol*, 2023 Oct; noad197, doi: 10.1093/neuonc/noad197.

Co-Autorschaften während des PhD-Programms:

2.)

Drexler R[#], Khatri R[#], Schüller U, **Eckhardt A**, Ryba AS, Sauvigny T, Dührsen L, Mohme M, Ricklefs T, Bode H, Hausmann F, Huber TB, Bonn S, Voß H, Neumann JE, Lamszus K, Gempt J, Westphal M, Heiland DH, Hänzelmann S[+], Ricklefs FL[+]. Temporal Change of DNA Methylation Subclasses Between Matched Newly Diagnosed and Recurrent IDH-Wildtype Glioblastoma. *Acta Neuropathol*. accepted for publication.

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<u>Andere Publikationen:</u>

14.)

Romano M, Bravin A, Mittone A, **Eckhardt A**, Barbone GE, Sancey L, Dinkel J, Bartzsch S, Ricke J, Alunni-Fabbroni M, Hirner-Eppeneder H, Karpov D, Giannini C, Bunk O, Bouchet A, Ruf V, Giese A, Coan P. A Multi-Scale and Multi-Technique Approach for the Characterization of the Effects of Spatially Fractionated X-ray Radiation Therapies in a Preclinical Model. *Cancers (Basel)*. 2021 Oct 1;13(19):4953. doi: 10.3390/cancers13194953. PMID: 34638437; PMCID: PMC8507698.

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11. Lebenslauf

entfällt aus datenschutzrechtlichen Gründen

12. Eidesstattliche Erklärung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....