Radiotherapy and Oncology 131 (2019) 66–74

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Original Article

Cancer genetic markers according to radiotherapeutic response in patients with primary glioblastoma – Radiogenomic approach for precision medicine

Kyungmi Yang [a,1], Sang Won Jung [b,c,1], Hyemi Shin [b,c,1], Do Hoon Lim [a,*,2], Jung-Il Lee [d], Doo-Sik Kong [d], Ho Jun Seol [d], Sung Tae Kim [e], Do-Hyun Nam [b,c,d,2]

[a] Department of Radiation Oncology, Samsung Medical Center, Sungkyunkwan University School of Medicine; [b] Institute for Refractory Cancer Research, Research Institute for Future Medicine, Samsung Medical Center; [c] Department of Health Sciences and Technology, Samsung Advanced Institute for Health Science & Technology, Sungkyunkwan University; [d] Department of Neurosurgery; and [e] Department of Radiology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 23 August 2018
Received in revised form 24 November 2018
Accepted 29 November 2018
Available online 31 December 2018

Keywords:
Glioblastoma
Radiotherapy
Response assessment
Biomarkers
Genome
Precision medicine

ABSTRACT

Background and purpose: To find genetic markers associated with response to radiotherapy (RT) in glioblastoma (GB) patients.

Materials and methods: From Jan 2009 to Dec 2016, 161 patients with newly diagnosed IDH-wild type GB were treated with surgery and adjuvant concurrent chemoradiotherapy with the Stupp's regimen, and then genomic research proceeded with their surgical specimens. Among the 161 patients, 49 with clinically measurable disease on postoperative MRI were analyzed. The response evaluation to RT was based on Response Assessment in Neuro-Oncology (RANO) criteria. For genomic analyses to compare between patients with progression and non-progression, Fisher test for DNA mutations and copy number alterations and the Gene Set Enrichment Analysis (GSEA) were performed.

Results: RT responses were non-progressive and progressive disease (PD) in 22 (44.9%) and 27 patients (55.1%), respectively. After three months, seven of PD exhibited pseudoprogression. For true response adjusting pseudoprogression from PD, 1-year progression-free survival for true Non-Responders (tNR-group) and true Responders (tR-group) were 0% and 45.4% ($p < 0.001$), and overall survival were 52.5% and 81.1% ($p = 0.046$), respectively. In genomic analyses, the tNR-group had more CDKN2A deletions (94.4% vs. 55.6%, $p = 0.013$), EGFR mutations (33.3% vs. 3.7%, $p = 0.012$) and less TP53 mutations (22.2% vs. 40.7%, $p = 0.333$) than the tR-group. In GSEA, immune-related gene sets were enriched in the tNR-group, and in contrast, some gene sets related with cell cycle were enriched in tR-groups.

Conclusion: The RANO criteria were feasible for the short-term response evaluation from RT despite of pseudoprogression. Genomic differences such as CDKN2A deletion, EGFR mutation, and immune- or inflammation-related related gene sets enrichment were found to be potentially predictive markers of RT.

Glioblastoma (GB) is the most common malignant type of brain tumor in adults. The standard treatment for GB is adjuvant concurrent chemoradiotherapy (CCRT) with temozolomide (TMZ) after tumor resection [1]. Despite therapeutic advances, the prognosis of GB remains very poor and certain patients show early progression in only several months, or even during treatment [2].

Cancer genomic statuses such as isocitrate dehydrogenase (IDH) mutation and O6-methylguanine DNA methyltransferase (MGMT) promoter methylation have been shown to indicate differential prognosis [3–6]. Furthermore, MGMT promoter methylation is known to be a predictive factor of response to TMZ [7,8]. However, there is still little evidence of specific markers to predict radiotherapeutic response. In addition, the Cancer Genome Atlas (TCGA) Research Network introduced the genomic subgroups of GB; proneural, neural, classical, and mesenchymal types [9]. In the study, more intensive therapy including CCRT and/or over 3 cycles of chemotherapy were beneficial in classical and mesenchymal types, but this result has not been used in treatment decisions including radiotherapy (RT).

* Corresponding author at: Department of Radiation Oncology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 81 Irwon-ro, Gangnam-gu, 06351 Seoul, Republic of Korea.
E-mail address: dh8.lim@samsung.com (D.H. Lim).

[1] Co-first authors: Kyungmi Yang, Sang Won Jung and Hyemi Shin contributed equally to this article.

[2] Co-corresponding authors: Do Hoon Lim and Do-Hyun Nam contributed equally to this article.

https://doi.org/10.1016/j.radonc.2018.11.025
0167-8140/

New and specific molecular markers for RT are needed for personalized treatment. Thus, the purpose of this study is to identify genetic markers associated with RT response in GB patients as a basic piece of knowledge for the development of precision medicine. Previous studies have attempted to identify treatment response based on genomic differences such as TCGA data. In contrast, in the present study, authors attempted to find these markers from RT response.

Patients and methods

Patients

From Jan 2009 to Dec 2016, 161 patients with newly diagnosed IDH-wild type GB were treated with craniotomy and tumor resection followed by adjuvant CCRT at Samsung Medical Center. Subsequently, their surgical specimens underwent genomic analysis. IDH status for all patients was checked with DNA sequencing. Eight of the patients were excluded – one pediatric patients age <18 years and 7 patients who did not undergo a postoperative brain MRI. Of the remaining 153 patients, a total of 49 patients with clinically measurable disease on postoperative MRI were analyzed. The definition of measurable disease is as follows: T1 contrast enhancing lesions with clearly defined margins on postoperative MRI, with two perpendicular diameters of at least 10 mm, visible on two or more axial slices [10]. This study was approved by the Institutional Review Board (IRB) of Samsung Medical Center with No. 2018-02-082-001 and written informed consent was waived by the IRB.

Treatment of glioblastoma

Subtotal resection (STR) was performed on all 49 patients. The pathologic diagnosis of GB was confirmed by specialized neuropathologists. Postoperative brain MRI was performed within 48 h after tumor resection in order to rule out the development of transient enhancement in the wall of the surgical cavity.

Adjuvant CCRT and adjuvant TMZ were prescribed for the patients [1]. For RT, a total dose of 60 Gy with 2 Gy per fraction was administered once daily, five days per week over a period of six weeks. The RT target was defined as follows: the gross tumor volume (GTV) as the surgical cavity plus residual gadolinium-enhancing lesions on postoperative MRI and clinical target volume as GTV plus a 1.5–2.0 cm margin. Concomitant chemotherapy with TMZ consisted of daily TMZ (75 mg per square meter of body-surface area per day, seven days per week from the first to the last day of RT), a break for four weeks, and then six cycles of adjuvant TMZ (150–200 mg per square meter for five days during each 28-day cycle). Follow-up brain MRI was obtained prior to adjuvant TMZ and after every other cycle of TMZ, or as needed when patients showed any neurological symptoms.

Evaluation for RT response

The response evaluation for RT was based on the Response Assessment in Neuro-Oncology (RANO) criteria. The RANO criteria for GB were described by Wen et al., where they were updated from the McDonald criteria in 2010 [10]. The assessment categories of the criteria are listed in Table 1. One radiation oncologist and one neurosurgeon evaluated the responses of all patients. If there were any differing opinions between the two physicians, an agreement was reached based on repeated review and discussion for each case. For radiological assessment, MRI at the time of three or four months after the end of CCRT was compared with baseline postoperative MRI. The flowchart of general steps for response evaluation is shown in Fig. 1.

Table 1
Characteristics for patients with measurable disease ($N = 49$).

Characteristics		No. of patients (%)
Age, years	Median (range)	57 (22–82)
	<50	14 (28.6%)
	≥50	35 (71.4%)
Sex	Male	30 (61.2%)
	Female	19 (38.8%)
Steroid use on CCRT	No	24 (49.0%)
	Yes	25 (51.0%)
Baseline ECOG PS	0–1	31 (63.3%)
	2–3	18 (36.7%)
MGMT methylation*	No	16 (32.7%)
	Yes	23 (46.9%)
	N/A	10 (20.4%)
RT dose (EQD2)	Median dose (range), Gy	60 (78 – 50)
	≥ 60 Gy	46 (93.9%)
	< 60 Gy	3 (6.1%)
Adjuvant TMZ after CCRT	Median cycles (range)	6 (0 – 6)
	6	30 (61.2%)
	2–4	13 (26.5%)
	0–1	6 (12.2%)

Abbreviations: CCRT, concurrent chemoradiotherapy; ECOG PS, the Eastern Cooperative Oncology Group performance status; MGMT, O6-methylguanine DNA methyltransferase; EQD_2, equivalent dose in 2 Gy; TMZ, temozolomide; N/A, Not-Available.

* The results of MGMT promotor methylation were obtained from methylation-specific polymerase chain reaction.

At first, using the results of response evaluation as RANO-response, we grouped patients with complete response (CR), partial response (PR), or stable disease (SD) as Responders (R-group) and patients with progressive disease (PD) as Non-Responders (NR-group). Pseudoprogression is the occurrence of transiently increased contrast enhancement following treatment without any actual disease progression. In order to identify pseudoprogression, we checked further follow-up MRI for an additional three months or more along with clinical events including re-operation, additional RT, or gamma knife surgery, as well as change of treatment for the next three months, which was regarded as the true progression. For adjusted analysis as true-response, we re-grouped patients with pseudoprogression from the NR-group to the R-group and renamed the adjusted two groups as true Responders (tR-group) and true Non-Responders (tNR-group).

Clinical progression was defined as any clinical event which was described as ruling out pseudoprogression or any evidence of intracranial or extracranial metastases during the entire follow-up period. Progression-free survival (PFS) or overall survival (OS) was the time from the date of surgical resection to the date of the first progression or death, or the latest follow-ups. The survival rates were estimated using the Kaplan–Meier method and compared using log-rank tests. Univariate and multivariate analyses with clinical and genomic factors were performed with log-rank tests and Cox regression analysis, respectively. A p value <0.05 was considered to be statistically significant in two-tailed tests. Statistical analysis was performed using SPSS software, standard version 20.0 (IBM Corporation, Armonk, NY, USA).

Genomic analyses

We performed genomic analyses with data from whole-exome sequencing (WES), targeted-exome sequencing, and RNA sequencing. The specimens were snap-frozen and preserved in liquid nitrogen until use. Genomic DNA and mRNA were extracted by using the DNeasy kit and the RNeasy kit (Qiagen, Hilden, Germany), respectively. Either the Illumina TruSeq Exome-capture kit or the

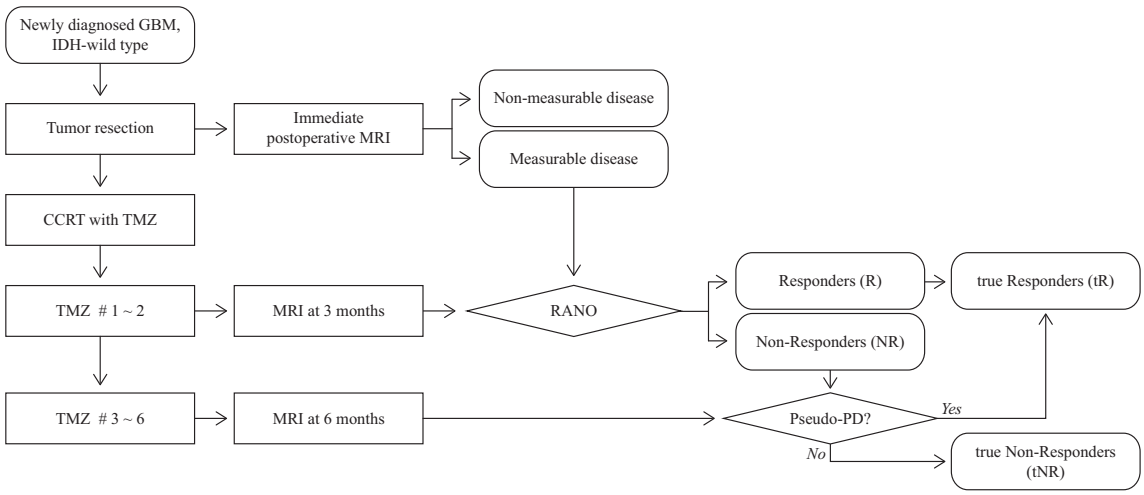

Fig. 1. Flowchart for treatment and the response evaluation for radiotherapy.

Agilent SureSelect kit was used for capturing exonic DNA fragments. For sequencing, Illumina HiSeq2000 was used to generate 2 * 101 bp paired-end reads.

The WES and target-exome sequencing reads in the FASTQ files were aligned to the human genome assembly using Burrows-Wheeler aligner. The initial alignment BAM files were preprocessed for sorting, removing duplicate reads, realigning reads around potential small indels, and recalibrating base quality score using SAMtools. MuTect and Somatic IndelDetector were used to make high-confidence predictions on somatic mutations from neoplastic and non-neoplastic tissue pairs. Variant Effector Predictor was used to annotate somatic mutations with potential functional consequences and other relevant information. Procedures for coverage calculation, mutation calling for tumors without the available matched normal, mutation validation. For Copy Number Alterations (CNA), we used the ngCGH python package version 0.4.4. The patient-matched normal WES data were used as the reference for calculating fold changes in copy number in tumors. In cases in which patient-matched normal data were not available, we created a 'pseudo-normal' profile to be used as the reference. The pseudo-normal profile is based on averaging a pool of 20 randomly chosen normal WES data, which were generated using the same sequencing platform and analysis pipeline as the tumor data. The R package DNAcopy (version1.30.0) was used to estimate DNA copy number for genomic segments. From the copy numbers at the segment level, the copy number for each gene was calculated by averaging the copy numbers of all exonic segments of the gene.

The RNA sequencing libraries were prepared using the Illumina TruSeq RNA Library Preparation Kit v2. The sequenced reads were trimmed and mapped onto hg19 using GSNAP version 2012-12-20 [11]. The resulting aligned reads were summarized into BED files using SAMtools and bedTools (bamToBed version 2.16.2) [12]. The BED files were used to estimate reads per kilobase of transcript per million reads (RPKM) using the R package DEGseq [13] and the RefSeq gene annotations (refFlat table, downloaded from the University of California, Santa Cruz [UCSC] Genome Browser, last accessed on August 6, 2012). For analysis of exon skipping, the untrimmed reads in FASTQ files were aligned on hg19 using GSNAP in the single-end mapping mode, while allowing splicing. The GSNAP results were parsed to isolate the "split" reads that span non-canonical splicing junctions (not annotated in the RefSeq or UCSC Known Gene database), with the minimal anchor of five nucleotides on each exon. If there were more than two such split reads between two exons, the event was called as a skipped exon event between the two exons. For analysis of point mutations, full-length sequencing reads in FASTQ files were aligned on hg19 using GSNAP with the same configurations as for skipped exon detection (single-end mapping mode allowing splicing), except for the output format: skipped exon analysis used a GSNAP output, while point mutation analysis used a SAM-formatted output. The SAM files were subjected to the same preprocessing procedures as the ones applied to the WES data, except that local realignments were restricted to exonic regions, so as not to confuse normal splicing events as misaligned indels. We identified potential point mutations using Unified Genotyper. The selected mutation candidates were annotated using VEP as with WES data.

Comparisons of genomics included DNA mutations such as single nucleotide variants, Insertions and Deletions, CNAs and Gene Set Enrichment Analysis (GSEA). The Fisher test was used to compare mutations and CNA with significant p values ≤ 0.05. Additionally, the GSEA score and the Enrichment Map were used to interpret and visualize gene expression [14]. The GSEA was conducted using Java with the Molecular Signatures Database (MSigDB; version 6.1) C2 gene set.

Results

The overall characteristics of the 49 patients with measurable disease on postoperative MRI are shown in Table 1. The median age of these patients was 57 years (range 22–82), with 35 patients (71.4%) being 50 years or over. There were 30 male patients (61.2%) and 19 female patients (38.8%). For baseline Eastern Cooperative Oncology Group (ECOG) performance status (PS), scores 0–1 and 2–3 were 31 (63.3%) and 18 (36.7%), respectively. The number of patients using steroids during CCRT was 25 (51.0%). The rate of MGMT promotor methylation was in 23 of available 39 patients from methylation-specific polymerase chain reaction (PCR) with biopsy or surgical specimens. All patients were treated with adjuvant CCRT with TMZ. For RT, median dose and fractions were 60 Gy in 30 fractions (range 78–50 Gy as equivalent dose in 2 Gy (EQD_2) in 15–33 fractions). In 3 patients (6.1%), less than 60 Gy was prescribed. For adjuvant TMZ, 6 cycles were completed in 30 patients (61.2%) and less than 2 cycles were in 6 patients (12.2%) due to progression or poor performance.

The therapeutic response evaluation for RT was based on RANO criteria. The individual evaluations of two physicians had relatively good agreement with 5 different responses in 49 patients (kappa = 0.795, $p < 0.001$). The final results of the evaluation are shown in Table 2. For radiological criteria, we generally compared

Table 2
Therapeutic response for patients with measurable disease ($N = 49$) at three months after radiotherapy.

Criteria		Response	No. of patients (%)
Radiological factors	1) T1 enhancement	CR/PR/SD	27 (55.1%)
		PD	22 (44.9%)
	2) T2 FLAIR	SD	36 (73.5%)
		PD	13 (26.5%)
	3) New out-field lesion(s)	No	46 (93.9%)
		Yes	3 (6.1%)
	1) + 2) + 3)	CR/PR/SD	25 (51.0%)
		PD	24 (49.0%)
Clinical factors	4) Steroid use	Stable or decreased	43 (87.8%)
		Increased	6 (12.2%)
	5) ECOG PS	Stable or improved	40 (81.6%)
		Worsened	9 (18.4%)
	4) + 5)	SD	37 (75.5%)
		PD	12 (24.5%)
RANO-response		R-group[a]	22 (44.9%)
		NR-group[b]	27 (55.1%)
True-response[c]		tR-group	29 (59.2%)
		tNR-group	20 (40.8%)

Abbreviations: CR/PR/SD, complete response/partial response/stable disease; PD, progressive disease; ECOG PS, the Eastern Cooperative Oncology Group performance status; RANO, Response Assessment in Neuro-Oncology.
[a] R-group included CR, PR, and SD.
[b] NR-group included only PD.
[c] Pseudoprogression was adjusted from RANO-response to true-response.

between the immediate postoperative MRI and posttreatment MRI at about three months after RT. In terms of T1 contrast enhancement on MRI, PD was seen in 22 patients (44.9%) and the others were at least SD. With T2 FLAIR, PD was seen in 13 patients (26.5%) and all patients except for one showed PD in T1WI. Of 22 PD in T1, 12 patients (54.5%) had PD in both T1 and T2 FLAIR images. New outfield enhancing lesions developed in three patients (6.1%) with one of these having no progression at primary lesion. Thus, radiological PD was seen in 24 patients (49.0%). In terms of clinical factors, there were six patients (12.2%) who required increased steroid doses and nine patients with worsened PS (18.4%). Of the patients with worsened PS, three also had increased steroid use. Therefore, clinical PD was seen in 12 patients (24.5%), and nine of these patients were with PD in radiological criteria while three were with no progression in images. As results of therapeutic response of RT with RANO criteria, CR/PR/SD (R-group) was in 22 patients (44.9%) and PD (NR-group) was in 27 patients (55.1%).

After further evaluation for a minimum of three months, seven of 27 patients with PD in RANO criteria exhibited pseudoprogression. They still had stable or partial responding conditions radiologically and clinically, with no evident progression. Thus, we decided that these seven patients were exhibiting pseudoprogression after RT and regrouped them from the NR-group to the tR-group. Of these seven patients, three were with clinical PD without radiological PD. As results of adjusted response evaluation, 29 patients (59.2%) were in the tR-group and 20 (40.8%) were in the tNR-group.

After a median follow-up period of 15 months (range 2–46), 90% of patients were considered to have experienced intracranial PD. Overall, 1-year PFS and OS rates were 25.8% and 69.6%, respectively, and median survival was 16 months. With RANO-response, 1-year PFS for R-group and NR-group were 43.2% and 12.5% ($p = 0.013$), and OS were 74.2% and 65.5% ($p = 0.349$), respectively (Fig. 2A and B). For excluding pseudoprogression from patients with PD, 1-year PFS for the tR- and tNR-group were 45.4% and 0 ($p < 0.001$), and OS were 81.1% and 52.5% ($p = 0.046$), respectively (Fig. 2C and D).

For genomic analysis, we compared both response groups; R versus NR, and tR versus tNR. As a result, in the groups from RANO-response, there was not any significant difference of gene mutation or CNA. So, we focused true-response, which also showed better fit for clinical outcomes. Of the 49 patients, DNA sequencing and RNA sequencing were analyzed in 45 and 41 samples, respectively, and at least one of the sequencings was done for each patient.

The comparisons of DNA sequencing were performed with all possible alterations in our database and the distribution of alterations for each sample is shown in Fig. 3A including TP53, EGFR, PTEN, PDGFRA, RB1, NF1, MDM2, CDKN2A as well as MGMT status and EGFRvIII. As a result, the tNR-group had more CDKN2A deletion and EGFR mutations with statistical significance and TP53 mutations seemed more frequent in the tR-group, but this trend was not statistically significant (Fig. 3; B and C). With CNAs, rates of high frequent samples with CDKN2A deletion were 94.4% and 55.6% in the tNR- and tR-group, respectively ($p = 0.013$). With mutation frequency analyses, the rates of EGFR mutation were 33.3% and 3.7%, and the rates of TP53 mutation were 22.2% and 40.7% in the tNR- and tR-group, respectively ($p = 0.012$ and 0.333). As for CDKN2A, EGFR, and TP53 from screening results, their correlation was analyzed with Fisher test. CDKN2A deletion and EGFR mutation had high concurrence and five samples of seven EGFR mutations were expressed with CDKN2A deletion. In contrast, TP53 alteration was significantly exclusive against CDKN2A ($p = 0.004$). MGMT status did not show any statistically different patterns with these markers including CDKN2A, EGFR, and TP53.

With patient characteristics, RT responses and genomic features, the results of univariate and multivariate analyses are shown in Table 3. True-response was the only significant factor for PFS and univariate analysis of OS. However, young age and MGMT methylation had better prognosis in multivariate analysis for OS ($p = 0.013$ and 0.034, respectively). Other genomic markers such as CDKN2A deletion, EGFR mutation, or TP53 mutation were not significant in PFS and OS (Table 4).

In GSEA, some gene sets related with cell cycle or cancer were enriched in the tR-group. In contrast, immune- or inflammation-related or hypoxia-related gene sets were enriched in the tNR-group (Fig. 4). However, specific immune suppressive or proinflammatory genes [15] were compared between the two groups, but there was no significance, except for TGF-ß. In detail with GB subtypes, the proneural type in the tR-group and classical or mesenchymal types in the tNR-group were significantly enriched from GSEA results (Supplement 1).

Discussions

The standard assessment criteria of treatment response for GB are the RANO Response Criteria [10], which was revised from Macdonald Criteria. The RANO Criteria consists of the radiologic change on CT or MRI and clinical deterioration such as increased steroid use or worse PS after treatment. It can be used for GB patients in clinics as well as in clinical trials. However, they are general guidelines and not specified for each treatment modality such as surgery, radiotherapy, chemotherapy, or target therapy. Furthermore, following adjuvant CCRT, the optimal time of evaluation has yet to be determined. Generally, the time of evaluation after treatment is recommended to be 12 weeks or later. For imaging evaluation, pseudoprogression was approximately 50% of early progression at the time of one-month follow-up after CCRT [16]. In addition, patients were treated with additional TMZ after CCRT as

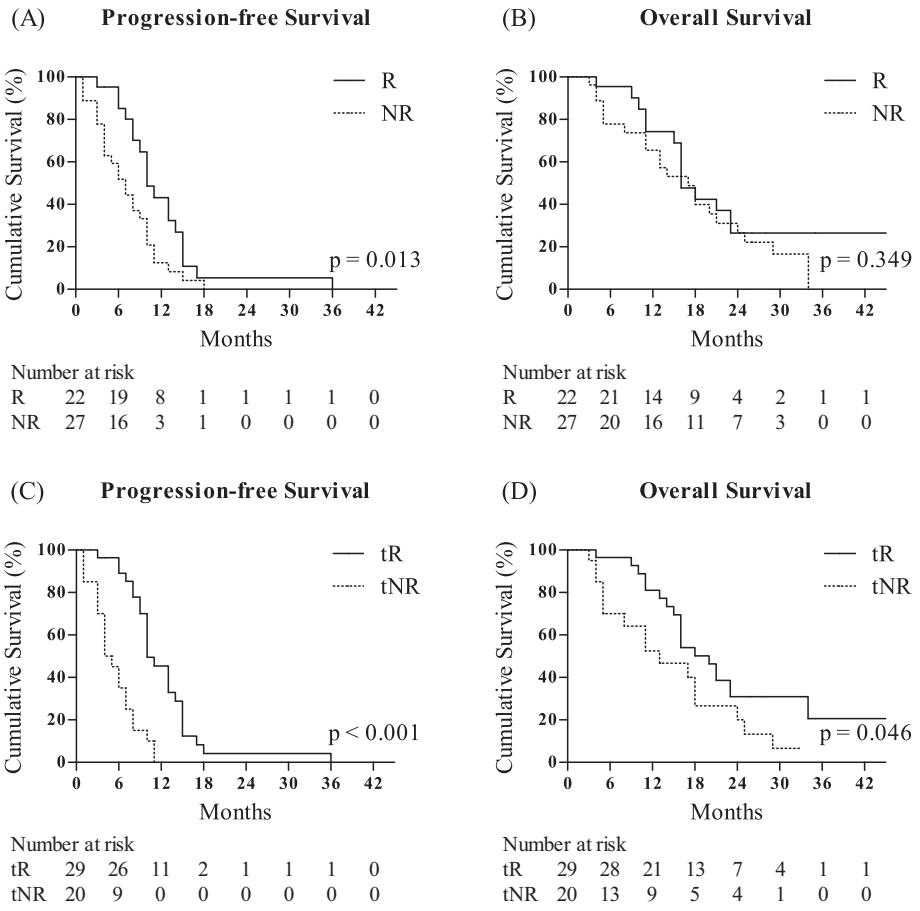

Fig. 2. Clinical outcomes between Responders (R-group) and Non-Responders (NR-group) with the RANO response (A and B), and between true Responders (tR-group) and true Non-Responders (tNR-group) with the adjustment for pseudoprogression from the RANO results (C and D).

the Stupp's regimen. As an appropriate time of response evaluation to minimize the pseudoprogression and the TMZ effect, three-month follow-up after RT was selected in this study. At this time, most patients were performed brain MRI as the routine protocol of our center.

The RANO criteria were feasible for evaluating the RT response and it was associated with progression. It has been reported that pseudoprogression was still prevalent in the first 12 weeks [16,17]. Similar to previous studies, there was 25.9% of pseudoprogression in PD patients of this study. Despite the rate of pseudoprogression, PFS was not much different between the initial evaluated groups (R- and NR-groups) and adjusted groups (tR- and tNR-groups). In addition, patterns of progression in the NR- or tNR-group were mostly with intracranial progression, which developed within one year. In the results of OS, however, the adjusted response from pseudoprogression showed better fit to survival. Therefore, the RANO criteria as the RT response evaluation tool in three months after RT might be useful for predicting the short-term progression.

For GB, genetic alterations including CDKN2A, EGFR, and TP53 are frequent and known to play key roles in tumorigenesis [18,19]. In this study, the alteration rates of CDKN2A, EGFR, and TP53 were associated with RT response. In addition, there was a pattern that CDKN2A deletion and EGFR mutation were expressed concurrently in patients showing progression after RT, and TP53 mutation was exclusively seen in responders. This pattern was similar with the general results from the TCGA database regardless of RT response, which was explained on the basis of tumorigenesis and genomic instability [20]. However, there is no definite evidence that this genomic pattern has an effect on clinical response including RT.

The CDKN2A is a cell cycle regulator gene which is located at chromosome 9 and codes for two tumor suppressors including p16INK4a and p14ARF [21]. P16 inhibits Cyclin D and cyclin-dependent kinase (CDK) 4 and 6, and sequentially controls RB at G1/S phase check point. In addition, p14ARF is an inhibitor of MDM2 which controls p53, and induces cell cycle arrest in the G2 phase and subsequent apoptosis. Deletion of the CDKN2A gene and amplification of the CDK6 are frequently reported in primary GB [22]. Some previous studies showed that the expression level of CDKN2A was correlated with survival in glioma cells [23]. So, GB patients with CDKN2A deletion have been expected to have poor prognosis with high proliferation and low apoptosis rates on the basis of its role and experimental evidence. Although the detailed mechanism remains unclear, it could be estimated from studies with the signal pathway. In targeting the Cyclin D-CDK4/6, Cyclin D was reported to be overexpressed after long-term and low-dose RT to tumor cells and to affect cell cycle progression and resistance to RT [24]. These results were not seen for GB, but CDKN2A deletion has the potential to aggravate the effect of Cyclin D accumulation with fractionated RT schedule as the standard treatment. The authors suggested that the high RT dose did not lead to Cyclin D accumulation and low dose with long-term RT exposure was associated with a low level of double stand breaks and genetic instability. Therefore, different RT schedule or higher dose RT may fit better to certain patients with this

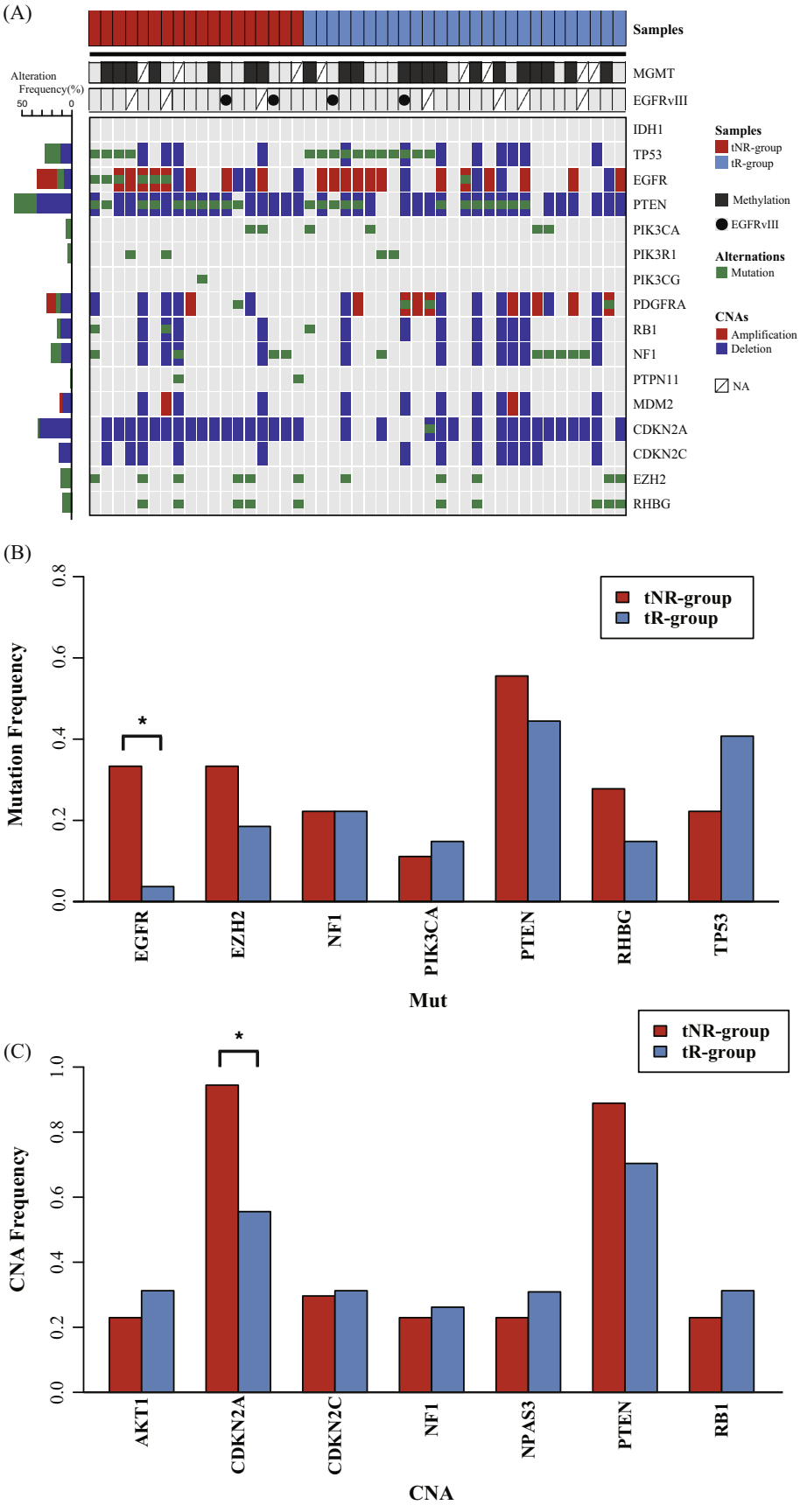

Fig. 3. Analyses of DNA sequencing; (A) the distribution of alterations, and comparisons of (B) Mutation Frequency and (C) Copy Number Alteration (CNA) Frequency in 45 samples. The star-marked parts are statistically significant (B and C). The p-value and false discovery rate (fdr) were 0.012 and 0.082 for EGFR mutation, 0.333 and 0.770 for TP53 mutation, and 0.014 and 0.100 for CDKN2A deletion, respectively. Additionally, the results of MGMT and EGFRvIII were obtained from methylation-specific polymerase chain reaction and RNA sequencing for comparison of the genomic distribution.

Table 3
Univariate and multivariate analysis with the clinical factors and the genomic markers ($N = 49$).

Variables		*n*	PFS				OS			
			Univariate		Multivariate*		Univariate		Multivariate*	
			1-yr (%)	*p*-Value	HR (95% CI)	*p*-Value	1-yr (%)	*p*-Value	HR (95% CI)	*p*-Value
Age, years	< 50	14	28.6	0.942	0.697	0.368	78.6	0.066	0.34	0.013
	≥ 50	35	24.6		(0.318–1.530)		66.1		(0.019–0.778)	
Sex	M	30	22.8	0.223	1.633	0.289	68.1	0.52	2.199	0.121
	F	19	30.8		(0.660–4.042)		72.2		(0.811–5.961)	
Steroid use on CCRT	No	24	26.7	0.543	1.213	0.596	72.5	0.282	0.731	0.487
	Yes	25	25.1		(0.595–2.472)		67.2		(0.302–1.769)	
Baseline ECOG PS	0–1	31	30.5	0.598	0.964	0.928	82.7	0.298	0.525	0.176
	2–3	18	17.8		(0.431–2.156)		48.1		(0.206–1.336)	
RANO-response	R	22	43.2	0.013	1.419	0.575	74.2	0.349	0.762	0.666
	NR	27	12.5		(0.418–4.811)		66.5		(0.222–2.616)	
True-response	tR	29	45.4	<0.001	0.187	0.007	81.1	0.046	0.672	0.522
	tNR	20	0		(0.056–0.628)		52.5		(0.199–2.268)	
MGMT methylation	Yes	16	29.7	0.124	0.738	0.498	81.6	0.357	0.3	0.034
	No	23	18.8		(0.306–1.788)		45		(0.099–0.910)	
CDKN2A del	Yes	32	32.7	0.294	1.048	0.918	80.6	0.603	1.18	0.766
	No	13	15.4		(0.428–2.568)		62.3		(0.396–3.513)	
EGFR mut	Yes	7	14.3	0.292	1.507	0.867	85.7	0.309	1.851	0.3
	No	38	30		(0.501–4.528)		74.4		(0.577–5.937)	
TP53 mut	Yes	13	30.5	0.984	0.964	0.924	73.8	0.16	0.511	0.189
	No	30	26		(0.389–2.338)		76.3		(0.188–1.391)	

Abbreviations: PFS, progression-free survival; OS, overall survival; CCRT, concurrent chemoradiotherapy; ECOG PS, the Eastern Cooperative Oncology Group performance status; RANO, Response Assessment in Neuro-Oncology; MGMT, O6-methylguanine DNA methyltransferase.
* Multivariate analysis was performed with 45 cases that had DNA sequencing.

Table 4
Summary of studies with radiotherapeutic response and TP53 alterations.

Authors, year	Type of studies	RT doses	Response or survival
Lang et al., 1996 [36]	In-vitro	9 Gy	Worse
Broaddus et al., 1999 [37]	In-vitro	10 Gy	Worse
Iwadate et al., 2001 [38]	In-vivo (rat)	15 Gy	Worse
Rubner et al., 2014 [40]	In-vitro	12 Gy	Worse
	In-vitro	10 Gy, 2 Gy/fx	No effect
Ruano et al., 2009 [39]	Clinical (CCRT)	N/A	Worse
Shiraishi et al., 2002 [42]	Clinical (RT or CCRT)*	60 Gy	No effect
BaxendineJone et al., 1997 [43]	Clinical (RT alone)	N/A	No effect
Tada et al., 1998 [41]	Clinical (RT alone)	N/A	Better

Abbreviations: CCRT, concurrent chemoradiotherapy; RT, Radiotherapy; N/A, Not-available.
* The result was not statistically significant and there was a gain of survival.

resistant feature to RT. However, dose-escalation has failed to show any clinical benefit [25]. For personalized strategy, therefore, patient selection and well-designed trial are needed.

Targeting CDKN2A-Cyclin D-CDK4/6-RB pathway has been considered for anti-tumor therapies. In a study of GB stem cells, AKT, Cyclin D, and CDK4 were upregulated and the inhibition of these signals induced re-sensitization to RT [26]. Cyclin D overexpression is mediated by pathway of AKT and AKT inhibitor was effective in suppressing radioresistance in animal studies [27]. Some clinical trials with AKT inhibitor such as API-2 have started for other tumors [28]. Also, CDK inhibitors such as palbociclib, which was approved for breast cancer from Food and Drug Administration, have been studied in-vitro and in-vivo for GB [29–31]. In the parts of these studies, they suggested that palbociclib had synergetic effect with RT. DNA damages and apoptosis in combined treated cells with palbociclib and RT were induced more than in palbociclib or RT alone. In a mouse model, similarly, combined treatment gained longer survivals. These results could be interpreted reversely that CDKN2A deletion might induce relative resistance for RT. Therefore, combined therapy with anti-tumor agents targeting the CDKN2A-CDK4/6-RB pathway is expected to improve clinical outcome for selected GB patients.

The EGFR mutation in this study was one of the worse predictive factors for RT, along with CDKN2A deletion. The EGFR is a gene or transmembrane protein activated by binding of growth factors and leads various functions such as cell proliferation, inhibition of apoptosis, angiogenesis, and inflammatory response. EGFR mutation or amplification is shown in 50% of GB, and EGFR amplifications including specialized form such as EGFRvIII are well known [20]. It has been considered to have negative effects on survival [18,32]. EGFR target agents have improved clinical outcomes and are widely used for various cancers, but there has been no clinical gain observed in GB patients. In this study, EGFR mutations were significantly high in non-responders, but the level of rate was similar to that generally reported. In contrast, they were rare in responders. These were all missense mutations and their functions are not fully known, but some of reported mutations expressed amplifications and might act like EGFRvIII [33]. However, EGFR amplification was not different between the two groups. Therefore, the effect of EGFR mutations in this study might be a poor predictive factor for RT response, but this still has weak evidence and requires further functional studies.

The p53, which was highly mutated in RT responders, is a tumor suppressor gene involved in regulation of the cell cycle, apoptosis, cell differentiation, or other cell regulations from DNA damages. In addition, TP53 is one of the frequent genetic alterations in the major pathway and pathogenesis of GB [34]. In general, TP53 alteration was identified in about 30% of GB. In this study, it was more

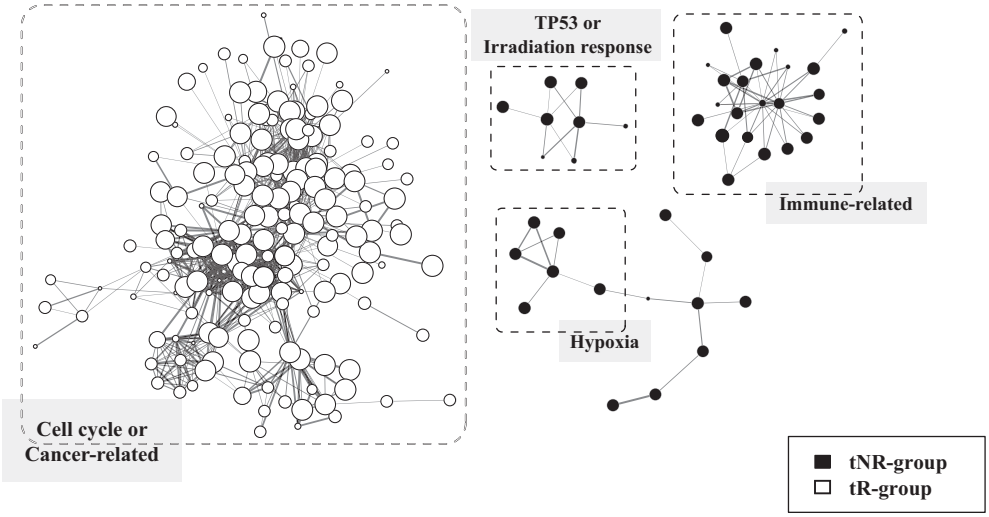

Fig. 4. GSEA Map in Responders (tR-group) and Non-Responders (tNR-group). The pathways with statistical values from GSEA were shown in Supplement 2.

than 40% in responders and higher than non-responders. TP53 mutation is generally known as a poor prognostic factor [35], which has resistance to apoptosis induced by DNA damage, but it is still controversial for GB patients with RT (Table 3). Some in-vitro and clinical studies have suggested that TP53 mutation showed RT resistant or worse clinical outcomes than TP53 wild-type of GB [36–38]. Similar results of studies were also found after recent intensive CCRT as the standard treatment [39]. In contrast, an in-vitro study suggested that fractionated RT, which was in more similar condition of treatment than in other in-vitro studies, stimulated G2 cell cycle arrest and immune response in even TP53 mutated cell lines and TP53 mutated cells were resistant to TMZ, not RT [40]. In addition, a study with adjuvant RT before CCRT era showed TP53 mutation was related to better survival [41]. Yet, there have been many studies with no significant effect of RT or CCRT in TP53 status [42,43] and meta-analyses also did not conclude the correlation between TP53 and overall clinical outcomes [44]. For weak or unclear correlations of TP53 with prognosis and response, there has been various hypotheses including the complex pathway or the effects of other regulators related with the TP53 pathway such as MDM2 and RB, and the heterogeneity of TP53 mutation [19,42,45]. With RT, we could suggest another hypothesis of exclusive mutation from the result of this study. TP53 mutation seemed to occur more often in responders without statistical significance, but this is possible to be explained as simply exclusive status against worse predictive factors.

As another part of genomic analysis, cell cycle-related gene sets in responders and immune- or inflammation-related or hypoxia-related gene sets in non-responders were highly enriched from GSEA. The result from responders seemed to have correlation with general mechanism of radiation effect to tumor. Also hypoxia, which is well-known cause inducing RT resistance in tumors [46], matched the result in non-responders. However, the association with immune or inflammation is unclear with worse RT response. Recently, there have been many studies which have suggested that RT may affect tumor and host immunity [47]. In addition, TCGA subtypes are suggested to be related with immune suppression and inflammation, especially in the mesenchymal subtype [15]. Referring that the rates of CDKN2A and EGFR alterations are high in the mesenchymal and classical subtypes [9], some of gene sets related with the two subtypes were significant in non-responders as well as immune suppressive or proinflammatory gene sets. With an additional analysis, however, TCGA subtype distribution was not different with RT response. Even though proneural type related with high TP53 mutation is known as having better prognosis or less effectiveness for aggressive therapy such as CCRT, there is no definite evidence of better RT response. Therefore, it is suggested that the functions of particular genes may affect RT response rather than the overall classification. Although the mechanism among immunity, tumor response, and subtypes remains unclear, recent studies are paying attention to immunotherapy with RT. Thus, further analysis related to immunity and RT response should be carried out following this study.

As limitations in this study, the number of patients with measurable disease was small and the data were reviewed retrospectively. Additionally, genomic differences were analyzed without functional and epigenetic factors except MGMT status from methylation-specific PCR. Also, we found the genomic markers related with RT response, but could not prove the impact of clinical outcome. Most patients were treated with TMZ after RT and long-term outcome might be a multifactorial result including both biological and therapeutic factors. We divided the samples into two groups including responders and non-responders, and vague responses such as stable disease or pseudoprogression were classified as responders. This could have affected the statistical results in small sized groups. So, a larger group of samples and more detailed measurement for RT response are needed in order to verify the results in this study.

In conclusion, RT response for GB with RANO criteria and the adjusted response with pseudoprogression were related with disease progression. The RANO criteria as the RT response evaluation tool in three months after RT might be useful for predicting short-term progression. Based on the RT response, clinically meaningful genetic difference was founded and CDKN2A deletion and EGFR mutation has a possibility to be worse predictive markers for RT. Additionally, immune-related RNA expression was enriched in patients with progression. Further studies with a larger number of patients for validation should follow in order to suggest molecular-based strategies, including RT, and to provide successful precision medicine for GB patients.

Conflict of interest

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry

Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI14C3418).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2018.11.025.

References

[1] Stupp R, Dietrich PY, Kraljevic SO, et al. Promising survival for patients with newly diagnosed glioblastoma multiforme treated with concomitant radiation plus temozolomide followed by adjuvant temozolomide. J Clin Oncol 2002;20:1375–82.
[2] McIntire K, Wong A. Meta-analysis of survival trends in glioblastoma in temozolomide treated patients from 2004 to 2016: no evidence for increase in survival benefit over time. Neuro-Oncology 2017;19:108.
[3] Li XZ, Li YB, Cao Y, et al. Prognostic implications of resection extent for patients with glioblastoma multiforme: a meta-analysis. J Neurosurg Sci 2017;61:631–9.
[4] Zhang K, Wang XQ, Zhou B, Zhang L. The prognostic value of MGMT promoter methylation in Glioblastoma multiforme: a meta-analysis. Fam Cancer 2013;12:449–58.
[5] Cheng HB, Yue W, Xie C, Zhang RY, Hu SS, Wang Z. IDH1 mutation is associated with improved overall survival in patients with glioblastoma: a meta-analysis. Tumor Biol. 2013;34:3555–9.
[6] Chen Y, Hu FL, Zhou YH, Chen WY, Shao HY, Zhang Y. MGMT promoter methylation and glioblastoma prognosis: a systematic review and meta-analysis. Arch Med Res 2013;44:281–90.
[7] Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987–96.
[8] Stupp R, Hegi ME, Mason WP, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 2009;10:459–66.
[9] Verhaak R. Integrated genomic analysis of glioblastoma: an update from the cancer genome atlas. Neuro-Oncology 2012;14:51-.
[10] Wen PY, Macdonald DR, Reardon DA, et al. Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group. J Clin Oncol 2010;28:1963–72.
[11] Wu TD, Nacu S. Fast and SNP-tolerant detection of complex variants and splicing in short reads. Bioinformatics 2010;26:873–81.
[12] Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 2010;26:841–2.
[13] Wang L, Feng Z, Wang X, Wang X, Zhang X. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics 2010;26:136–8.
[14] Merico D, Isserlin R, Stueker O, Emili A, Bader GD. Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. PLoS ONE 2010;5:e13984.
[15] Doucette T, Rao G, Rao A, et al. Immune heterogeneity of glioblastoma subtypes: extrapolation from the cancer genome atlas. Cancer Immunol Res 2013;1:112–22.
[16] Gladwish A, Koh ES, Hoisak J, et al. Evaluation of early imaging response criteria in glioblastoma multiforme. Radiat Oncol 2011;6:121.
[17] Wen PY, Chang SM, Van den Bent MJ, Vogelbaum MA, Macdonald DR, Lee EQ. Response Assessment in Neuro-Oncology Clinical Trials. J Clin Oncol 2017;35:2439–49.
[18] Karsy M, Huang T, Kleinman G, Karpel-Massler G. Molecular, histopathological, and genomic variants of glioblastoma. Front Biosci (Landmark Ed) 2014;19:1065–87.
[19] Karsy M, Neil JA, Guan J, Mahan MA, Colman H, Jensen RL. A practical review of prognostic correlations of molecular biomarkers in glioblastoma. Neurosurg Focus 2015;38:E4.
[20] Furnari FB, Fenton T, Bachoo RM, et al. Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev 2007;21:2683–710.
[21] Pavletich NP. Mechanisms of cyclin-dependent kinase regulation: structures of Cdks, their cyclin activators, and Cip and INK4 inhibitors. J Mol Biol 1999;287:821–8.
[22] Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. Science 2008;321:1807–12.
[23] Liu WD, Lv GH, Li YW, Li L, Wang B. Downregulation of CDKN2A and suppression of cyclin D1 gene expressions in malignant gliomas. J Exp Clin Cancer Res 2011;30.
[24] Shimura T, Fukumoto M, Kunugita N. The role of cyclin D1 in response to long-term exposure to ionizing radiation. Cell Cycle 2013;12:2738–43.
[25] Badiyan SN, Markovina S, Simpson JR, et al. Radiation therapy dose escalation for glioblastoma multiforme in the era of temozolomide. Int J Radiat Oncol Biol Phys 2014;90:877–85.
[26] Shimura T, Noma N, Oikawa T, et al. Activation of the AKT/cyclin D1/Cdk4 survival signaling pathway in radioresistant cancer stem cells. Oncogenesis 2012;1:e12.
[27] Shimura T, Kakuda S, Ochiai Y, et al. Acquired radioresistance of human tumor cells by DNA-PK/AKT/GSK3beta-mediated cyclin D1 overexpression. Oncogene 2010;29:4826–37.
[28] Feun LG, Blessing JA, Barrett RJ, Hanjani P. A phase II trial of tricyclic nucleoside phosphate in patients with advanced squamous cell carcinoma of the cervix. A Gynecologic Oncology Group Study. Am J Clin Oncol 1993;16:506–8.
[29] Michaud K, Solomon DA, Oermann E, et al. Pharmacologic inhibition of cyclin-dependent kinases 4 and 6 arrests the growth of glioblastoma multiforme intracranial xenografts. Cancer Res 2010;70:3228–38.
[30] Hashizume R, Zhang A, Mueller S, et al. Inhibition of DNA damage repair by the CDK4/6 inhibitor palbociclib delays irradiated intracranial atypical teratoid rhabdoid tumor and glioblastoma xenograft regrowth. Neuro-Oncology 2016;18:1519–28.
[31] Whittaker S, Madani D, Joshi S, et al. Combination of palbociclib and radiotherapy for glioblastoma. Cell Death Discov 2017;3:17033.
[32] Taylor TE, Furnari FB, Cavenee WK. Targeting EGFR for treatment of glioblastoma: molecular basis to overcome resistance. Curr Cancer Drug Targets 2012;12:197–209.
[33] Xu H, Zong H, Ma C, et al. Epidermal growth factor receptor in glioblastoma. Oncol Lett 2017;14:512–6.
[34] Chin L, Meyerson M, Aldape K, et al. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 2008;455:1061–8.
[35] Montgomery RM, Queiroz LD, Rogerio F. EGFR, p53, IDH-1 and MDM2 immunohistochemical analysis in glioblastoma: therapeutic and prognostic correlation. Arq Neuropsiquiatr 2015;73:561–8.
[36] Lang FF, Yung WK, Raju U, Libunao F, Terry NH, Tofilon PJ. Enhancement of radiosensitivity of wild-type p53 human glioma cells by adenovirus-mediated delivery of the p53 gene. J Neurosurg 1998;89:125–32.
[37] Broaddus WC, Liu Y, Steele LL, et al. Enhanced radiosensitivity of malignant glioma cells after adenoviral p53 transduction. J Neurosurg 1999;91:997–1004.
[38] Iwadate Y, Mizoe JE, Osaka Y, Yamaura A, Tsujii H. High linear energy transfer carbon radiation effectively kills cultured glioma cells with either mutant or wild-type p53. Int J Radiat Oncol Biol Phys 2001;50:803–8.
[39] Ruano Y, Ribalta T, de Lope AR, et al. Worse outcome in primary glioblastoma multiforme with concurrent epidermal growth factor receptor and p53 alteration. Am J Clin Pathol 2009;131:257–63.
[40] Rubner Y, Muth C, Strnad A, et al. Fractionated radiotherapy is the main stimulus for the induction of cell death and of Hsp70 release of p53 mutated glioblastoma cell lines. Radiat Oncol 2014;9.
[41] Tada M, Matsumoto R, Iggo RD, et al. Selective sensitivity to radiation of cerebral glioblastomas harboring p53 mutations. Cancer Res 1998;58:1793–7.
[42] Shiraishi S, Tada K, Nakamura H, et al. Influence of p53 mutations on prognosis of patients with glioblastoma. Cancer 2002;95:249–57.
[43] BaxendineJones J, Campbell I, Ellison D. p53 status has no prognostic significance in glioblastomas treated with radiotherapy. Clin Neuropathol 1997;16:332–6.
[44] Jin YL, Xiao WZ, Song TT, Feng GJ, Dai ZS. Expression and Prognostic Significance of p53 in Glioma Patients: A Meta-analysis. Neurochem Res 2016;41:1723–31.
[45] Costa B, Bendinelli S, Gabelloni P, et al. Human glioblastoma multiforme: p53 reactivation by a novel MDM2 inhibitor. PLoS One 2013;8:e72281.
[46] Rockwell S, Dobrucki IT, Kim EY, Marrison ST, Vu VT. Hypoxia and radiation therapy: past history, ongoing research, and future promise. Curr Mol Med 2009;9:442–58.
[47] Chow KK, Hara W, Lim M, Li G. Combining immunotherapy with radiation for the treatment of glioblastoma. J Neurooncol 2015;123:459–64.