Assessment of circulating tumor DNA in cerebrospinal fluid by whole exome sequencing to detect genomic alterations of glioblastoma

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Abstract

Background: Cerebrospinal fluid (CSF) has been demonstrated as a better source of circulating tumor DNA (ctDNA) than plasma for brain tumors. However, it is unclear whether whole exome sequencing (WES) is qualified for detection of ctDNA in CSF. The aim of this study was to determine if assessment of ctDNA in CSF by WES is a feasible approach to detect genomic alterations of glioblastoma.

Methods: CSFs of ten glioblastoma patients were collected pre-operatively at the Department of Neurosurgery, Sun Yat-sen University Cancer Center. ctDNA in CSF and genome DNA in the resected tumor were extracted and subjected to WES. The identified glioblastoma-associated mutations from ctDNA in CSF and genome DNA in the resected tumor were compared.

Results: Due to the ctDNA in CSF was unqualified for exome sequencing for one patient, nine patients were included into the final analysis. More glioblastoma-associated mutations tended to be detected in CSF compared with the corresponding tumor tissue samples (3.56 ± 0.75 *vs.* 2.22 ± 0.32, $P = 0.097$), while the statistical significance was limited by the small sample size. The average mutation frequencies were similar in CSF and tumor tissue samples ($74.1\% \pm 6.0\%$ *vs.* $73.8\% \pm 6.0\%$, $P = 0.924$). The R132H mutation of isocitrate dehydrogenase 1 and the G34V mutation of H3 histone, family 3A (H3F3A) which had been reported in the pathological diagnoses were also detected from ctDNA in CSF by WES. Patients who received temozolomide chemotherapy previously or those whose tumor involved subventricular zone tended to harbor more mutations in their CSF.

Conclusion: Assessment of ctDNA in CSF by WES is a feasible approach to detect genomic alterations of glioblastoma, which may provide useful information for the decision of treatment strategy.

Keywords: Circulating tumor DNA; Cerebrospinal fluid; Glioblastoma; Mutation; Whole exome sequencing

Introduction

Glioblastoma (GBM) is the most prevalent primary malignant brain tumor with bleak prognosis in adults.[1] Despite the standard of care therapy, the median survival duration is only 14.6 months.[2] The diagnosis of GBM has stepped into a molecular era since the 2016 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS) incorporates molecular parameters into the classification of CNS tumor entities.[3] Some of these molecular parameters have a prognostic role for GBM patients.[4,5] Meanwhile, these molecular parameters may also serve as predictive biomarkers of therapeutic effect, including extent of surgical resection and sensitivity of chemotherapy.[6-8] Therefore, the early diagnosis of molecular parameters may be helpful for the decision of treatment strategy.

Circulating tumor DNA (ctDNA) is tumor-derived fragmented DNA circulating in body fluid and may reflect the entire tumor genome. Cerebrospinal fluid (CSF) directly contacts the brain tumor and has been demonstrated as a better source of ctDNA than plasma for brain tumors.[9,10] Recent studies have shown that ctDNA detected using targeted deep sequencing in CSF could represent the genomic alterations of brain tumors[10] and monitor the evolution of the glioma genome.[9] However, most of these previous results were based on Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable

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Cancer Targets, a custom sequencing-based tumor sequencing assay that captures all protein-coding exons of 341, 410, or 468 cancer-associated genes with deep coverage.[11] However, this assay is not so accessible and affordable as whole exome sequencing (WES) overall the world. However, it is unclear whether WES is qualified for detection of ctDNA in CSF.

Here, we aimed to determine if assessment of ctDNA in CSF by WES is a feasible approach to detect genomic alterations of GBM.

Methods

Ethical approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Medical Ethics Committees of Sun Yat-sen University Cancer Center (No. GZR2018-244), followed the 1964 *Helsinki Declaration* and its later amendments or comparable ethical standards. Informed consents to review medical data and use biological samples were obtained from all patients before the study.

Study cohort and sample collection

Ten patients who were diagnosed with GBM and underwent lumbar puncture pre-operatively as part of their clinical evaluation for neurological signs or symptoms and surgical resection were included in this study. The study subjects were enrolled at the Department of Neurosurgery, Sun Yat-sen University Cancer Center. Collection of CSF was performed through lumbar puncture. To avoid the contamination of blood, only the clear CSF was collected. After spinning down, cell-free supernatants were collected and stored at −80°C. Then, all patients underwent surgical resection and fresh tumor tissue samples were stored in liquid nitrogen. The diagnosis of each patient was confirmed by pathologist according to the 2016 WHO Classification of Tumors of the CNS. Clinicopathological data including patient's age at diagnosis, sex, number of lesions, size of lesions, subventricular zone (SVZ) involvement described by Lim *et al*,[12] isocitrate dehydrogenase 1 (*IDH1*) mutation status, O (6)-methylguanine-DNA methyltransferase (MGMT) promoter methylation status, 1p/19q chromosomal status, telomerase reverse transcriptase (TERT) promoter mutation status, H3 histone, family 3A (*H3F3A*) mutation status, and Histone cluster 1, H3b (HIST1H3B) mutation status were collected from medical records.

Extraction of ctDNA and genome DNA

For each patient, 5 to 10 mL of CSF was subjected to ctDNA extraction using QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA, USA). Genome DNA from the resected tumor was extracted using a DNeasy Blood and Tissue Kit (Qiagen). DNA concentration was quantified by the Qubit 4.0 Fluorometer (Invitrogen, Singapore). Bioanalyzer agilent 2100 (Agilent Technologies, Palo Alto, CA, USA) defined the sample DNA qualities with high sensitivity DNA chip.

Exome-sequencing analysis

Exome sequencing libraries were prepared with the SureSelect Human All Exon 50 Mb Targeted exome enrichment kit v4, targeting all protein-coding exons, exon-intron boundaries, and untranslated regions of all protein-coding genes. The library quality and quantity were controlled by KAPA SYBR FAST LightCycler 480 qPCR kit (KAPA Biosystems, Wilmington, MA, USA), on BioAnalyzer agilent 2100 High Sensitivity DNA chip (Agilent Technologies). Libraries containing captured DNA fragments were then sequenced on the Illumina HiSeq 2500 system as paired-end 150 bp. The mean sequencing depth was 93X for all samples.

Single nucleotide variants (SNVs) and small insertions and deletions (INDELs) calling

First, raw Fastq reads were trimmed with Trimmomatic as previously described,[13] reads were then aligned in paired-end mode to the human genome build 19 (hg19) version using Burrow-Wheeler Aligner-maximal exact match (BWA-MEM) with default parameters to generate a binary sequence alignment map file.[14] SNVs were called with MuTect, a Bayesian framework for the detection of somatic mutations, and small INDELs (<30 bp in length) were detected by SomaticINDELDetector, a tool in GATK version 2.3.9, according to the pipeline previously described.[11,15,16] Variants identified within CSF and tumor tissue were filtered out if found in the 1000 Genomes East Asian cohort (1000g2014oct_eas), avsnp150, cosmic or esp6500sic2_all (NHLBI-ESP project) databases. Finally, we only kept functional variants predicted as damaging by Polyphen2, LRT, MutationTaster, FATHMM and Sift for further analysis.[17-20]

Statistical analyses

SPSS 22.0 (IBM Corp, Armonk, NY, USA). USA) was used for statistical analyses. The independent Student's *t* test was used to assess the statistical significance between any two preselected groups. The results were presented as mean ± standard deviation (SD). A two-sided $P < 0.05$ was considered as statistically significant.

Results

Patients' clinicopathological characteristics

Because the ctDNA was unqualified for exome sequencing for one patient, nine patients were included into the final analysis. The clinicopathological characteristics of patients were summarized in Table 1. Median age at diagnosis was 58 years (range, 16–74 years). Only one patient was female who was diagnosed with recurrent GBM and had received surgery followed by radiotherapy plus concomitant and adjuvant temozolomide (TMZ) before CSF collection. All of the eight male patients were diagnosed with primary therapy-naïve GBM. Two patients had multiple lesions. The median maximum diameter of lesions was 5.1 cm. One patient had the tumor involving SVZ. In pathological diagnosis, R132H mutation of *IDH1* and G34V of *H3F3A* were identified in one patient, respectively. C228T mutation

Table 1: Clinicopathological characteristics of nine glioblastoma patients.

Patient	GBM01	GBM02	GBM03	GBM04	GBM05	GBM06	GBM07	GBM08	GBM09
Age at diagnosis (years)	58	74	64	44	16	62	55	63	51
Sex	Male	Male	Male	Female	Male	Male	Male	Male	Male
Classification	Primary	Primary	Primary	Recurrent	Primary	Primary	Primary	Primary	Primary
Number of lesions	Single	Single	Single	Single	Multiple	Single	Single	Multiple	Single
Maximum diameter of lesions (mm)	71	51	63	55	46	34	67	33	37
SVZ involvement	No	No	No	No	No	No	No	Yes	No
IDH1/2 mutation status	Wildtype	Wildtype	Wildtype	*IDH-1* Mutation	Wildtype	Wildtype	Wildtype	Wildtype	Wildtype
MGMT promoter status	Methylated	Unmethylated	Methylated	Methylated	Unmethylated	Methylated	Unmethylated	Unmethylated	Unmethylated
1p/19q chromosomal status	19q deletion	Intact	Intact	Intact	Intact	1p/19q co-deletion	19q deletion	1p/19q co-deletion	19q deletion
TERT promoter mutation status	C228T mutation	Unknown	C228T mutation	Wildtype	Wildtype	Wildtype	C228T mutation	Unknown	C228T mutation
H3F3A mutation status	Wildtype	Wildtype	Wildtype	Wildtype	G34V mutation	Wildtype	Wildtype	Wildtype	Wildtype
HIST1H3B mutation status	Wildtype	Wildtype	Wildtype	Wildtype	Wildtype	Wildtype	Wildtype	Wildtype	Wildtype

H3F3A: H3 histone, family 3A; HIST1H3B: Histone cluster 1; IDH: Isocitrate dehydrogenase; MGMT: O(6)-methylguanine-DNA methyltransferase; SVZ: Subventricular zone; TERT: Telomerase reverse transcriptase.

Table 2: GBM-associated genes defined by the Catalogue of Somatic Mutations In Cancer (COSMIC) and the Cancer Genome Atlas (TCGA)-GBM database.

Genes	Ensembl ID
FGFR1	ENSG00000077782
FGFR3	ENSG00000068078
ERBB4	ENSG00000178568
STK11	ENSG00000118046
KIT	ENSG00000157404
PTEN	ENSG00000171862
FBXW7	ENSG00000109670
IDH2	ENSG00000182054
PIK3CA	ENSG00000121879
IDH1	ENSG00000138413
PIK3R1	ENSG00000145675
EGFR	ENSG00000146648
MAP2K1	ENSG00000169032
NF1	ENSG00000196712
MET	ENSG00000105976
TP53	ENSG00000141510
RB1	ENSG00000139687
KDR	ENSG00000128052
ATRX	ENSG00000085224
NRAS	ENSG00000213281
TSC1	ENSG00000165699
NTRK1	ENSG00000198400
PDGFRA	ENSG00000134853
H3F3A	ENSG00000163041
JAK2	ENSG00000096968
MTOR	ENSG00000198793
SMARCA4	ENSG00000127616

ATRX: ATRX chromatin remodeler; EGFR: Epidermal growth factor receptor; ERBB4: Erb-B2 receptor tyrosine kinase 4; FBXW7: F-Box and WD repeat domain containing 7; FGFR1: Fibroblast growth factor receptor 1; FGFR3: Fibroblast growth factor receptor 3; GBM: Glioblastoma; H3F3A: H3 histone, family 3A; IDH1: Isocitrate dehydrogenase 1; IDH2: Isocitrate dehydrogenase 2; JAK2: Janus kinase 2; KDR: Kinase insert domain receptor; KIT: KIT proto-oncogene receptor tyrosine kinase; MAP2K1: Mitogen-activated protein kinase kinase 1; MET: MET proto-oncogene receptor tyrosine kinase; MTOR: Mechanistic target of rapamycin kinase; NF1: Neurofibromin 1; NRAS: NRAS proto-oncogene GTPase; NTRK1: Neurotrophic receptor tyrosine kinase 1; PDGFRA: Platelet derived growth factor receptor alpha; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PIK3R1: Phosphoinositide-3-kinase regulatory subunit 1; PTEN: Phosphatase and tensin homolog; RB1: RB transcriptional corepressor 1; SMARCA4: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4; STK11: Serine/threonine kinase 11; TP53: Tumor protein P53; TSC1: TSC complex subunit 1.

of TERT promoter and MGMT promoter methylation were identified in four patients, respectively. Deletion of chromosome arm 19q was detected in three patients and 1p/19q co-deletion occurred in two patients. Nobody had HIST1H3B-mutant tumor.

Landscape of genetic alterations in CSF and tumor tissues

Deep sequencing that achieved a coverage of approximately 74× in CSF and over 100× in tumor identified and validated averagely 1211 non-silent variants per patient (range 62–7482). Across these nine pairs of CSF and

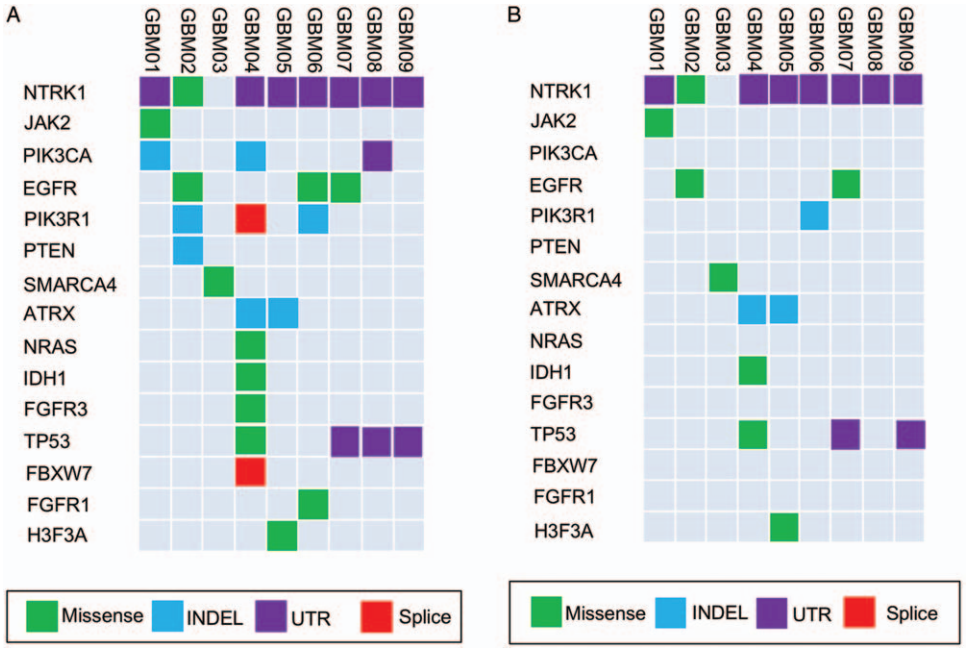

Figure 1: Landscape of GBM-associated mutations detected in CSF (A) and tumor tissue (B). ATRX: ATRX chromatin remodeler; CSF: Cerebrospinal fluid; EGFR: Epidermal growth factor receptor; FBXW7: F-box and WD repeat domain containing 7; FGFR1: Fibroblast growth factor receptor 1; FGFR3: Fibroblast growth factor receptor 3; GBM: Glioblastoma; H3F3A: H3 histone, family 3A; INDEL: Insertion and deletion; IDH1: Isocitrate dehydrogenase 1; JAK2: Janus kinase 2; NRAS: NRAS proto-oncogene, GTPase; NTRK1: Neurotrophic receptor tyrosine kinase 1; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic Subunit Alpha; PIK3R1: Phosphoinositide-3-Kinase Regulatory Subunit 1; PTEN: Phosphatase And Tensin Homolog; SMARCA4: SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 4; TP53: Tumor Protein P53; UTR: Untranslated region.

corresponding tumor tissue samples, a total of 10,690 SNVs and 212 INDELs were called. The exons and surrounding noncoding genomic regions of 27 protein-coding genes that were most frequently mutated in GBM defined by the Catalogue of Somatic Mutations In Cancer (COSMIC) and the Cancer Genome Atlas (TCGA)-GBM database were captured [Table 2]. After excluding those identified in matched germline DNA, the non-silent coding variants (missense, nonsense, short INDELs, splicing variants) of 15 GBM-associated genes were enriched in CSF [Figure 1A] and the corresponding tumor tissue samples [Figure 1B]. We have identified novel SNVs and INDELs in epidermal growth factor receptor (*EGFR*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), phosphoinositide-3-kinase regulatory subunit 1 (*PIK3R1*), and fibroblast growth factor receptor 3 (*FGFR3*), which are the major genes of the receptor tyrosine kinase (RTK)/Ras GTPase/phosphatidylinositol 3-kinase (PI3K) pathway, and *IDH1*, ATRX chromatin remodeler (ATRX), and tumor protein P53 (*TP53*), genes well known to be altered in GBM. A detailed table including the identified non-silent coding variants in both CSF and tumor tissues was listed [Table 3].

In general, more glioblastoma-associated mutations tended to be detected in CSF comparing with the corresponding tumor tissue samples (3.56 ± 0.75 *vs.* 2.22 ± 0.32, $P = 0.097$), while the statistical significance was limited by the small sample size. The average mutation frequencies were similar in CSF and tumor tissue samples ($74.1\% \pm 6.0\%$ *vs.* $73.8\% \pm 6.0\%$, $P = 0.924$). In patient GBM01, PIK3CA mutation was only detected in CSF but not in the corresponding tumor tissue sample. In patient GBM02, PIK3R1 mutation and phosphatase and tensin homolog (*PTEN*) mutation were uniquely detectable in the CSF. In patient GBM04, PIK3CA mutation, neuroblastoma RAS viral oncogene homolog (*NRAS*) mutation, FGFR3 mutation, PIK3R1 mutation, and F-Box and WD repeat domain containing 7 (*FBXW7*) mutation were all exclusively found in the CSF. In the CSF of patient GBM06, we found the *EGFR* mutation and *FGFR1* mutation which were undetectable in the corresponding tumor tissue sample. Similarly for patient GBM08, PIK3CA mutation and TP53 mutation were only detectable in the CSF. Moreover, patient GBM04 who received TMZ chemotherapy previously and patient GBM08 whose tumor involved SVZ harbored the biggest number of SNVs and INDELs in their CSF [Figure 2]. Most of mutations identified in patient GBM04 had higher mutation frequencies in CSF than tumor (except K93R of *EGFR*). Meanwhile, the R132H mutation of *IDH1* which had been reported in the pathological diagnosis of patient GBM04 was also detected in both CSF and tumor by WES. Likewise, the G34V mutation of *H3F3A* which had been reported in the pathological diagnosis of patient GBM05 was detected a higher mutation frequency in CSF than tumor.

Discussion

In this study, we found that gene mutations in GBM could be effectively detected in CSF ctDNA by WES pre-operatively. *IDH1* R132H mutation and *H3F3A* G34V mutation which had been respectively reported in the pathological diagnoses of patient GBM04 and patient GBM05 were detected in their CSF and confirmed in the corresponding tumor tissue samples by WES. It has reported that *IDH1* mutant GBM is more amenable to surgical resection and has a survival benefit associated with maximal surgical resection.[6] The determination of gene

Table 3: Non-silent mutational profiles in the CSF and tumor tissues.

Genes	Chr	Position	Mutation	CSF (%)	Tumor (%)	Patient
NTRK1	1	156785617	5′UTR	54.0	51.5	GBM01
JAK2	9	5044432	G127D	31.5	39.5	GBM01
PIK3CA	3	178957861	5710insA	37.5	0	GBM01
EGFR	7	55221830	V247M	36.8	84.2	GBM02
NTRK1	1	156811945	R28W	48.7	52.7	GBM02
PIK3R1	5	67584513	51delT	33.3	0	GBM02
PTEN	10	89725294	65delT	28.6	0	GBM02
SMARCA4	19	11121110	R726H	90.1	68.5	GBM03
PIK3CA	3	178957862	5710delA	38.2	0	GBM04
PIK3R1	5	67569842	Splicing	50.0	0	GBM04
ATRX	X	76938092	E848fs	77.8	53.1	GBM04
NRAS	1	115258747	G12D	32.6	0	GBM04
IDH1	2	209113112	R132H	38.1	31.3	GBM04
FGFR3	4	1808278	S567F	57.7	0	GBM04
TP53	17	7578535	K93R	79.2	85.1	GBM04
NTRK1	1	156785617	5′UTR	100	100	GBM04
FBXW7	4	153244302	Splicing	33.3	0	GBM04
ATRX	X	76889195	V1605fs	95.5	89.7	GBM05
NTRK1	1	156785617	5′UTR	100	100	GBM05
H3F3A	1	226252156	G34V	47.7	40.0	GBM05
PIK3R1	5	67589589	E88fs	46.7	39.1	GBM06
EGFR	7	55221822	A244V	34.5	0	GBM06
FGFR1	8	38272308	K567E	48.8	0	GBM06
NTRK1	1	156785617	5′UTR	99.0	100	GBM06
EGFR	7	55221822	A244V	94.9	97.7	GBM07
TP53	17	7578645	5′UTR	100	100	GBM07
NTRK1	1	156785617	5′UTR	42.6	44.2	GBM07
PIK3CA	3	178957861	3′UTR	46.7	0	GBM08
TP53	17	7578645	5′UTR	100	0	GBM08
NTRK1	1	156785617	5′UTR	100	100	GBM08
TP53	17	7578645	5′UTR	100	100	GBM09
NTRK1	1	156785617	5′UTR	100	100	GBM09

ATRX: ATRX Chromatin Remodeler; CSF: Cerebrospinal fluid; EGFR: Epidermal Growth Factor Receptor; FBXW7: F-Box And WD Repeat Domain Containing 7; FGFR1: Fibroblast Growth Factor Receptor 1; FGFR3: Fibroblast Growth Factor Receptor 3; H3F3A: H3 histone, family 3A; IDH1: isocitrate dehydrogenase 1; JAK2: Janus Kinase 2; NRAS: NRAS Proto-Oncogene, GTPase; NTRK1: Neurotrophic Receptor Tyrosine Kinase 1; PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; PIK3R1: Phosphoinositide-3-Kinase Regulatory Subunit 1; PTEN: Phosphatase And Tensin Homolog; SMARCA4: SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 4; TP53: Tumor Protein P53; UTR: Untranslated region.

mutations pre-operatively may facilitate individualized surgical strategies for GBM in the future.

The standard tissue biopsy is the traditional way to determine gene mutations in GBM. However, this method is limited by the difficult access to the tumor and especially the tumor heterogeneity. The molecular heterogeneity within tumors has been increasingly recognized.[21-23] Intriguingly, we found that more gene mutations were detected in CSF ctDNA than in tumor tissue samples, which indicated that CSF ctDNA was better to represent the tumor genome than tumor tissue samples.

Compared with other patients, the numbers of gene mutations detected in CSF ctDNA were extremely higher for Patient 4 and Patient 8. Since Patient 4 suffered recurrent GBM, we speculated the hypermutation of Patient 4 was induced by the treatment of TMZ before CSF collection.[24,25] As the tumor of Patient 8 had involved ventricle, the hypermutation in CSF ctDNA may be due to the direct exposure of tumor into CSF.

The main limitation of this study was the small sample size. Although we found more GBM-associated mutations were detected in CSF comparing with the corresponding tumor tissue samples, the difference was not statistically significant. Future studies with large sample size are warranted to confirm these preliminary results.

In summary, assessment of ctDNA in CSF by WES is a feasible approach to detect genomic alterations of GBM, which may provide useful information for the decision of treatment strategy.

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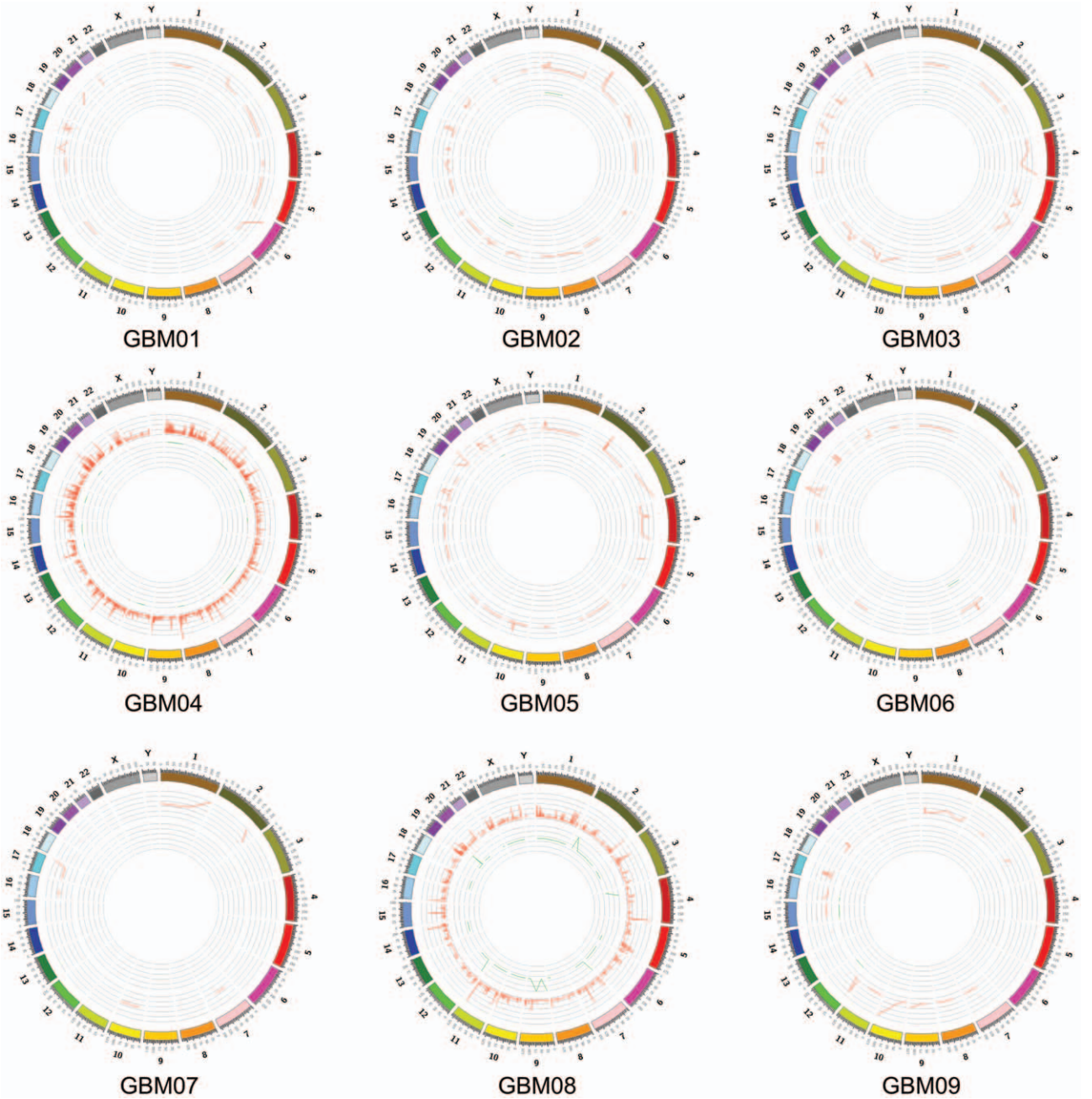

Figure 2: Landscape of total SNVs and INDELs detected in cerebrospinal fluid of all patients. GBM: Glioblastoma; INDELs: Insertions and deletions; SNVs: Single nucleotide variants.

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Conflicts of interest

None.

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