Tumor DNA from tumor in situ fluid was used to track genomic status and evolutionary models of gliomas under first-line treatment

Jinliang Yu
Children's Hospital Affiliated to Zhengzhou University, Henan Children's Hospital, Zhengzhou Children's Hospital
Sensen Xu
Zhengzhou University People's Hospital, Henan University People's Hospital
Zhiyuan Sheng
Zhengzhou University People's Hospital, Henan University People's Hospital
Yushuai Gao
Zhengzhou University People's Hospital, Henan University People's Hospital
Chaojie Bu
Zhengzhou University People's Hospital, Henan University People's Hospital
Shuang Wu
Zhengzhou University People's Hospital, Henan University People's Hospital
Kaiyuan Deng
Zhengzhou University People's Hospital, Henan University People's Hospital
Yage Bu
Zhengzhou University People's Hospital, Henan University People's Hospital
Guanzheng Liu
Zhengzhou University People's Hospital, Henan University People's Hospital
Zhaoyue Yan
Zhengzhou University People's Hospital, Henan University People's Hospital
Yulong Jia
Zhengzhou University People's Hospital, Henan University People's Hospital
Guangzhong Guo
Zhengzhou University People's Hospital, Henan University People's Hospital
Ziyue Zhang
Zhengzhou University People's Hospital, Henan University People's Hospital
Lingfei Kong
Henan Provincial People's Hospital, People's Hospital of Zhengzhou University
Gang Liu
Zhengzhou University People's Hospital, Henan University People's Hospital

Meiyun Wang
Zhengzhou University People's Hospital, Henan University People's Hospital, Henan Medical University
Chunxiao Ma
Zhengzhou University People's Hospital, Henan University People's Hospital
Tianxiao Li
Zhengzhou University People's Hospital, Henan University People's Hospital
Xingyao Bu (xingyaob@zzu.edu.cn)
Zhengzhou University People's Hospital, Henan University People's Hospital

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Research Article

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Abstract

Background: Tumor DNA from tumor in situ fluid (TISF) provides a minimally invasive approach to repeatedly interrogated the glioma genome and provides an opportunity to monitor the dynamics of pressure-induced cloning for recurrence and treatment selectivity. Understanding the evolution of glioma will be of great help in clinical and scientific research of glioma. Currently, no study has explored the recurrence process of glioma.

Methods: We performed high-depth generation gene sequencing in multiple regions of matched primary and recurrent tumors. Using 60 primary tumor tissues and their TISF, 26 of whom had tumor recurrence and 6 underwent 2 surgeries, we analyzed the heterogeneity of each period.

Results: We measured considerable genomic variation in glioma patients at different times under the stress of first-line treatment. In gliomas dominated by genomic alleles with low frequency (VAF < 1%), radiographic residues had higher VAF ($p = 0.016$), and patients with postoperative recurrence also had higher VAF ($p < 0.0001$). Under the pressure of treatment, polyclonal mutations gradually increased with tumor evolution, and high-frequency dominant clones gradually appeared. Samples of relapsed TISF contained more abundant clonal mutations. Sequencing of relapsed tumor tissue and relapsed TISF samples showed high consistency in mutation detection and estimation of allele frequency ($p < 0.0001$, VAF correlation, $R^2 = 0.8737$). Heterogeneity at different stages of the tumor was shown in patients who were continuously monitored. We determined that TISF may detect elevated Tumor DNA variant allele fraction prior to positive imaging findings and effectively identify patients with pseudoprogression.

Conclusions: Continuous TISF-DNA analysis reveals the evolutionary structure of gliomas under first-line therapy and can be used to track postoperative residual disease and tumor recurrence. Most importantly, it could move glioma research forward.

Introduction

Glioma is the most common primary intracranial malignancy and there is no standard treatment for recurrence[1]. Recent advances have shown that there is significant epigenetic and environmental heterogeneity within gliomas, which are linked together to lead to extreme phenotypic heterogeneity at the cellular level, providing multiple therapeutic resistance mechanisms, and complicating the treatment of gliomas for recurrence[2].

Enhanced detection of minimal residual disease by targeted sequencing of phased variants in circulating tumor DNA[3], guiding adjuvant immunotherapy[4],tumor early relapse detection[5], tracking the evolutionary dynamics and heterogeneity of tumors[6], Positive ctDNA is considered as possible evidence of early cancer molecular residual disease (MRD)[7-10]. The clinical correlation between ctDNA and cancer is the direction of future clinical decision making. Nevertheless, the related research in glioma is seriously insufficient. Due to the special anatomical location of gliomas, it is difficult to detect effective cfDNA

fragments in the blood. CSF-ctDNA is only positive when the tumor load is high[11-13], which loses the genomic evolution information of gliomas under the treatment pressure. TISF-DNA as a biomarker of glioma load is our latest discovery[14]. It overcomes the shortcomings of tissue-based biomarkers, enables rapid sampling and multiple continuous monitoring to detect tumor heterogeneity in a timely manner. We found that TISF-DNA was more sensitive to low tumor load, while the TISF collection process was even less invasive than lumbar puncture, and TISF was superior to cerebrospinal fluid (CSF) even when gliomas were adjacent to CSF systems[15]. We found not only a higher positive rate of TISF-DNA in the early postoperative period, but also a higher concentration of cfDNA than CSF, even in paired samples tested at the same time[16]. However, continuous monitoring is needed to understand the evolution of postoperative glioma recurrence, and relevant studies are still insufficient at present.

We conducted a prospective and observational study to monitor the temporal heterogeneity of gliomas under first-line therapy using continuous TISF-DNA testing and investigate its potential relevance to clinical outcomes.

Methods

Study approval

Approval was obtained from the Ethics Committee and Institutional Review Committee of the People's Hospital of Henan Province. The procedures used in this study adhere to the tenets of the Declaration of Helsinki. All methods are carried out according to the approved guidelines. Obtain written informed consent from all patients.

Patients and sample collection

This retrospective cohort study was conducted on January 1, 2016 at the People's Hospital of Henan Province on January 31, 2022. TISF-DNA was obtained using the same method as in our previous study[14]. The primary tumor samples were obtained by surgery, and the NCCN regimen was used to guide the treatment after surgery. TISF samples were collected at different postoperative times: 35d, 36-120d, and more than 120d after surgery(tumor progression according to RANO standard, T1 enhancement increased by ≥ 25%, T2/FLAIR increased, and new lesions and clinical manifestations deteriorated). Six patients underwent a second surgical resection of the tumor after radiographic recurrence was found. Fresh tumor tissue comes from surgical resection, and HE staining specimens contain more than 70% of tumor cells, which neuropathologists has confirmed. The average depth of targeted sequencing of tumor tissue was 500X, the average depth of paired blood sequencing was 250X, and the average depth of TISF-DNA sequencing was 20000X. Grade III-IV glioma patients were followed up every 4-6 weeks and grade II glioma patients were followed up every 2-3 months. All patients underwent MRI at each follow-up evaluation.

Targeted sequencing analysis of Tumor-associated DNA

All clinical TISF samples and tumor tissue samples were detected by Next generation sequencing. QIAamp DNA Tissue and Blood Kit for Genomic DNA (Qiagen; Germantown, MD, USA) extract. TISF sample and blood sample were centrifuged in EDTA tube at 1900 g for 10 min, and the precipitate particles were frozen at −80°C. The supernatant was centrifuged at 16000 g for 10 min and transferred to −80°C for preservation. CfDNA was extracted from TISF and blood supernatant using Mag-MAX CellFree DNA isolation kit (Thermo Fisher Scientific, Waltham, MA, USA). Finally, all segregated DNA were quantified using the Qubit 2.0 Fluorometer with the Qubit dsDNA HS Assay kit (Life Technologies; Carlsbad, CA, USA).

As described elsewhere, the isolated DNA was cut into 150-200 bp fragments using Covaris M220 Focused-ultrasonicator™ Instrument (Covaris; Woburn, MA, USA). Following the manufacturer's direction9,10 to construct Fragmented DNA and ctDNA libraries with the KAPA HTP Library Preparation Kit (Illumina platforms; KAPA Biosystems; Wilmington, MA, USA). The DNA libraries were captured with a designed panel of 68 genes for brain tumors (GenetronHealth; Beijing, China), these containing major brain tumor-related genes. The DNA sequencing was based on novaseq high-throughput sequencing platform. After sequencing, we adopted such criteria that a mutation had an allele fraction of $\geq$ 0.1% and a total of $\geq$ 4 reads were considered existing in liquid samples. Known recurrent loci were further manually checked with Integrative Genomics Viewer (IGV v2.3.34) in the target sample comparing to the normal blood DNA. Using the dbNSFP and the Exome Aggregation Consortium (ExAC) database to exclude either benign mutations with pp2_hdiv score < 0.452 or polymorphic nonsynonymous mutations sites. At the end, all detected mutations were annotated for genes using ANNOVAR, Oncotator and Vep.

Statistical analysis

Graph drawing was completed in GraphPad Prism(Version 8.0C), heat maps were generated by TBtools, and statistical tests were performed by SPSS (Version 23.0; Armonk, NY, IBM Corp), Fisher's exact test for categorical variables, Wilcoxon test and Mann-Whitney(rank-sum) test or Kruskal-Wallis test for continuous variables.

Results

Genomic landscapes of glioma at various stages under first-line treatment

Sequencing data from primary tumors and TISF-DNA in glioma patients, The genome of primary tumor (n = 60) represents the characteristics of glioma before surgery, 0-35d after surgery (n = 20) represents the genomic characteristics of minimal residual disease in tumor lumen at the early stage after surgical resection, 36-120d after surgery (n = 19) represents the genomic characteristics of glioma undergoing chemotherapy (low grade) and chemoradiotherapy (high grade), and 120d after surgery (n = 38) represents genomic characteristics of further chemotherapy during treatment, and radiographic tumor recurrence was found in 26 patients at the time of sampling.

In general, the genomes are different at different stages of the treatment process, but there is some similarity. For the primary tumor (Figure 1A), the mutation rates were TP53 (44%), IDH1 (39%), PTEN (24%), CIC (17%), EGFR (17%), NF1 (13%), ATRX (11%), NOTCH1 (11%), PIK3CA (11%), PIK3R1 (11%). At the stage of minimal residual disease 0-35d after surgery (Figure 1B), mutation rates were higher in NF1 (45%), SETD2 (45%), CIC (40%), TP53 (40%), BRCA2 (30%), GNAS (30%), PIK3R1 (30%), PTEN (30%), TSC1 (30%), TSC2 (30%). After treatment 36-120 days after surgery (Figure 1C), mutation rates were higher in NF1 (53%), TP53 (53%), TSC2 (47%), PTCH1 (42%), BRCA1 (37%), FAT1 (37%), BRCA2 (32%), EGFR (32%), NOTCH1 (32%), SETD2 (32%). 120d after surgery (FIG. 1D), the mutation rates were TP53 (37%), SETD2 (39%), IDH1 (26%), NF1 (26%), NOTCH1 (26%), RELA (24%), EGFR (16%), FAT1 (21%), GNAS (21%), TP53 (37%), SETD2 (39%), IDH1 (26%), NF1 (26%), NOTCH1 (26%). TSC2 (21%). For radiographic recurrence (FIG. 1E), the mutation rates were TP53 (58%), NF1 (38%), SETD2 (38%), EGFR (35%), FAT1 (31%), PTEN (31%), RELA (31%), TSC2 (31%), BCOR (27%), CIC (27%). Gliomas at different stages were highly heterogeneous during postoperative treatment, with only 15.75% shared mutation rate and 84.25% private mutation rate (Figure 1F).

Genomic VAF changes under first-line treatment

We found that VAF(Variant Allele Fraction, In the primary tumor tissue, there was such a high VAF(Figure 2A),97.37% of the clones had a VAF value greater than 1%, and only 2.27% of the low-frequency clones. However, in the early stage after tumor resection (within 35d), 87.84% of the low-frequency clones had a VAF value less than 1% (Figure 2B), and only 12.16% of the clones with a VAF value greater than 1% With further postoperative treatment (36-120 days), mutations with VAF greater than 1% increased slightly (Figure 3C) to 14.29% 120 days after surgery, clone mutations with VAF greater than 1% were significantly increased, accounting for 30.42%(Figure D). In TISF samples from patients with recurrence, clone mutations with VAF greater than 1% were found to be slightly higher overall, accounting for 30.84% (Figure E, Figure G, $p < 0.0001$). Overall, tumor recurrence was associated with an increased frequency of genomic cloning after glioma surgery (Figure F, G). In addition, we also found that patients with higher VAF within 35 days after surgery may be associated with postoperative residual. Imaging showed that patients with significant residual VAF were larger than those with complete radiographic resection (Figure 2H, $p = 0.016$), while patients with radiographic recurrence during postoperative treatment had higher VAF than those without recurrence (Figure 2H, $p < 0.0001$, $p < 0.0001$).

Changes in specific gene types in glioma under first-line treatment

Polyclonal mutations, MMR-related mutations of mismatch repair genes (MSH2, MSH3, MSH6, MLH1, MLH3, PMS1 and PMS2) were summarized. The accumulation of G:C>A:T (transitions at non-CPg sites in hypermutated gliomas after exposure to alkylating Agents[17,18]), and VAF greater than 5% of the genome changes (Figure 3). In general, Polyclonal mutation, MMR related mutation and gene clone with VAF > 5% increased gradually in gene clone with postoperative treatment and tumor progression after glioma resection, and the proportion of patients also increased gradually. Temozolomide-associated hypermutation did not detect secondary phenomena in gene clones and patients (Figure 3C, 3D).

Therefore, MMR and Polyclonal mutations may be associated with glioma recurrence after treatment, which is consistent with previous studies.

Genomic characterization of glioma patients was continuously monitored

TISF samples from 15 patients in this study were obtained by continuous monitoring, TISF-1 (postoperative 35 days),TISF-2 (postoperative 36-120 days), and TISF-3 (postoperative 120 days), and they were divided into two groups: non-recurrence group (n = 4) and recurrence group (n = 11). Similar to the above conclusions, low frequency distribution was observed in both groups at the early stage after glioma resection (Figure 4A,4B), but increased frequency of some genes in TISF-3 was observed in the recurrence group, while not in the non-recurrence group With the postoperative progression of glioma, there were more private mutations in TISF-DNA than in the primary tissue (Figure 4C), with a maximum of 156 mutations in TISF-3 Mutations shared between TISF and primary tissue decreased gradually, with the highest in TISF-1 and decreased with tumor progression (Figure 4D). A lower proportion of mutations shared between TISF-1 and TISF-3 was also found in TISF-DNA samples (Figure 4E). This suggests that the minimal residual disease in the early postoperative stage may be closer to the primary tumor, while the tumor at recurrence is more heterogeneous with the primary tumor.

Six patients underwent surgical resection after radiographic recurrence, and 129 gene loci in the recurrent TISF were not detected in the recurrent tissue (Figure 5A), and only 32 loci were detected in the recurrent tissue. There were only 17 identical mutation loci in the primary tumor and the recurrent tumor, with a consistency rate of only 30.36%. There was no significant correlation between the frequency of mutations in the genes consistent with the primary and recurrent tissues (Figure 5B, $P = 0.5912$, $R^2 = 0.01468$), and even there was no significant correlation between the frequency of mutations shared between TISF samples from 26 recurrent patients and their tissues (Figure. 5C, $P = 0.8987$, $R^2 = 0.0004102$), but a positive correlation was found between the frequency of shared mutations in relapsed tissues and relapsed TISF (Figure 5D, $P < 0.0001$, $R^2 = 0.8737$). Shared mutant genes (Figure 5F1-F6), most of which were up-regulated and only 3 loci were down-regulated (Figure 5E). Patients with private mutated genes in primary and recurrent TISF (n = 5, Figure. 5H1-H5) were significantly different in gene frequency (Figure 5G), indicating the heterogeneity of tumor evolution under therapeutic pressure.

Clinical relevance of genomic changes in glioma

Based on the results of this study, we summarized the correlation between genomic changes after glioma surgery and clinical practice. We found that the elevation of VAF predates radiographic findings (Figure 6A, Patient 24, patient 31, and patient 34), indicating that tumor DNA-relapse may be present when radiographic findings are not positive and is an ultra-early manifestation of relapse. We performed a second tumor resection for 6 patients with radiological manifestations of recurrence, and only 3 patients were found to have pathological manifestations of tumor recurrence, with polyclonal mutations and high VAF values in their recurrent TISF. Two patients presented with extensive inflammatory and necrotic tissue, and no clones were detected in their TISF (Figure 6B). Finally, we summarized the evolution model

in the case of glioma in the first-line treatment (Figure 6C), the first primary tumors is surgical resection, and its leading cloning is cleared, the postoperative residual disease stages exist a large number of low frequency and cloning of mutation, in the treatment of postoperative line pressure part gene mutation in cloning advantage frequency increased, the imaging may not appear at this time a positive result, As the tumor evolved further, it became more and more obvious that the dominant clone would lead to tumor recurrence.

Discussion

Although tumor biopsy remains the gold standard for glioma diagnosis, liquid biopsy based on TISF-DNA overcomes many of the limitations of tumor biopsy and has clinical advantages over circulating tumor DNA derived from blood and cerebrospinal fluid[16]. Biopsy is sampled locally at a single metastatic site, which may introduce sampling bias[19]. Biopsies can be painful and cause anxiety; And biopsies carry the risk of bleeding or infection. CtDNA is almost impossible to extract from the blood of glioma patients. CSF-ctDNA is a good method, but it is almost difficult to detect in the early stage after tumor resection. Positive CSF-ctDNA often requires certain conditions, including large tumor load, tumor progression, and tumor diffusion into the ventricular system or subarachnoid space[11-13]. This makes it impossible for us to detect genomic changes at the early stage of glioma evolution, let alone detect tumor recurrence at the tumor DNA level earlier than imaging, making early postoperative targeted therapy more difficult. In this study, we attempted to understand the genomic changes of glioma under the pressure of first-line treatment, so as to provide more meaningful therapeutic guidance for clinical treatment of glioma.

Our analysis of glioma driver gene heterogeneity, clonicity, and recurrence provides important insights into genomic state at all stages of glioma first-line treatment. First, in the first-line treatment context, the primary clones of the tumor genome differ at different stages (Figure 1A-1D). In the early stage after surgical resection, the gene status was dominated by low-frequency subclonal mutations, and no tumor-dominated clones appeared at this time. As the treatment progresses, tumor polyclonal mutations increase and high-frequency mutations increase, leading to the emergence of tumor-dominated clones (Figure 3C, 3D), which eventually leads to tumor recurrence. In addition, we observed that mutations detected in recurrent tumor tissue samples were in good agreement with matched TISF samples (Figure 5D), demonstrating the reliability of TISF-DNA in detecting somatic variation during systemic treatment of gliomas. However, the tissue samples of recurrent TISF differed greatly from those of the primary tumor, resulting in poor consistency of mutation frequency (Figure 5C), as well as large differences in private mutations (Figure 5G), indicating the heterogeneity of recurrent gliomas.

Our study also found that TISF-DNA found increased tumor DNA VAF levels, but there was no positive imaging performance at this time (Figure 6A). At this time, the tumor had DNA level recurrence, which has been confirmed by relevant studies, and we found this for the first time in glioma[10,20-22]. In addition, the TISF of patients with recurrence showed high frequency clonal mutations similar to that of the recurrent tumor tissue, while the TISF of patients with pseudoprogression showed low frequency clonal mutations and tissue test was negative, but they all showed positive findings on imaging. This indicates that TISF-

DNA can identify pseudoprogression of glioma in clinical practice, thus providing therapeutic guidance for physicians. Yet no similar study has been done.

Conclusion

Our results show the state and evolution patterns of the genome under pressure from first-line treatment of glioma. We determined that continuous TISF can be used to track postoperative residual disease, early tumor-DNA relapse, and pseudoprogression of gliomas. It can dynamically monitor the genomic changes and progress of glioma in real time, monitor the spatio-temporal heterogeneity of glioma, provide guidance for the clinical management of glioma patients, promote the development of glioma-related research, and ultimately contribute to significant progress in prolonging the life of patients.

Declarations

Ethical Approval and Consent to participate

The studies involving human participants were reviewed and approved by the Institutional Review Board (IRB) and Ethics Committee of Henan Provincial People's Hospital (Zhengzhou, China). All methods were carried out in accordance with the approved guidelines. Written informed consent was obtained from all patients. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Consent for publication

Not applicable.

Availability of supporting data

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://figshare.com/articles/dataset/Data/19203251.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JLY, XYB and TXL designed research; ZYS, YSG, CJB, WS, YGB, KYD, ZYY and YLJ contributed new reagents/analytic tools; ZYZ, GZG, GZL, GL, LFK, MYW and CXM analyzed data; and JLY, XYB, SSX and CXM wrote the paper. All authors read and approved the final manuscript.

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Authors' information

[1]Department of Neurosurgery, Children's Hospital Affiliated to Zhengzhou University, Henan Children's Hospital, Zhengzhou Children's Hospital, Zhengzhou, China. [2]Department of Neurosurgery, Juha International Central for Neurosurgery, Henan Provincial People's Hospital, Zhengzhou University People's Hospital, Henan University People's Hospital, Zhengzhou, 450003, China. [3]Henan Provincial Neurointerventional Engineering Research Center, Henan International Joint Laboratory of Cerebrovascular Disease, and Henan Engineering Research Center of Cerebrovascular Intervention Innovation, Zhengzhou, Henan, 450003, China. [4]Department of Psychological Medicine, Henan Provincial People's Hospital, Zhengzhou University People's Hospital, Henan University People's Hospital, Zhengzhou, Henan, 450003, China. [5]Department of Cerebrovascular disease, Henan Provincial People's Hospital, Zhengzhou University People's Hospital, Henan University People's Hospital, Zhengzhou, Henan, 450003, China. [6]Department of Center for Clinical Single Cell Biomedicine, Clinical Research Center, Department of Oncology, Henan Provincial People's Hospital,Zhengzhou University People's Hospital, Henan University People's Hospital, Zhengzhou, 450003, China. [7]Department of Radiology, Henan Provincial People's Hospital, Zhengzhou University People's Hospital, Henan University People's Hospital, Henan Medical University, Zhengzhou, 450003, China. [8]Department of Pathology, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, No. 7 Weiwu Road, Zhengzhou, 450003, Henan, China.

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Figures

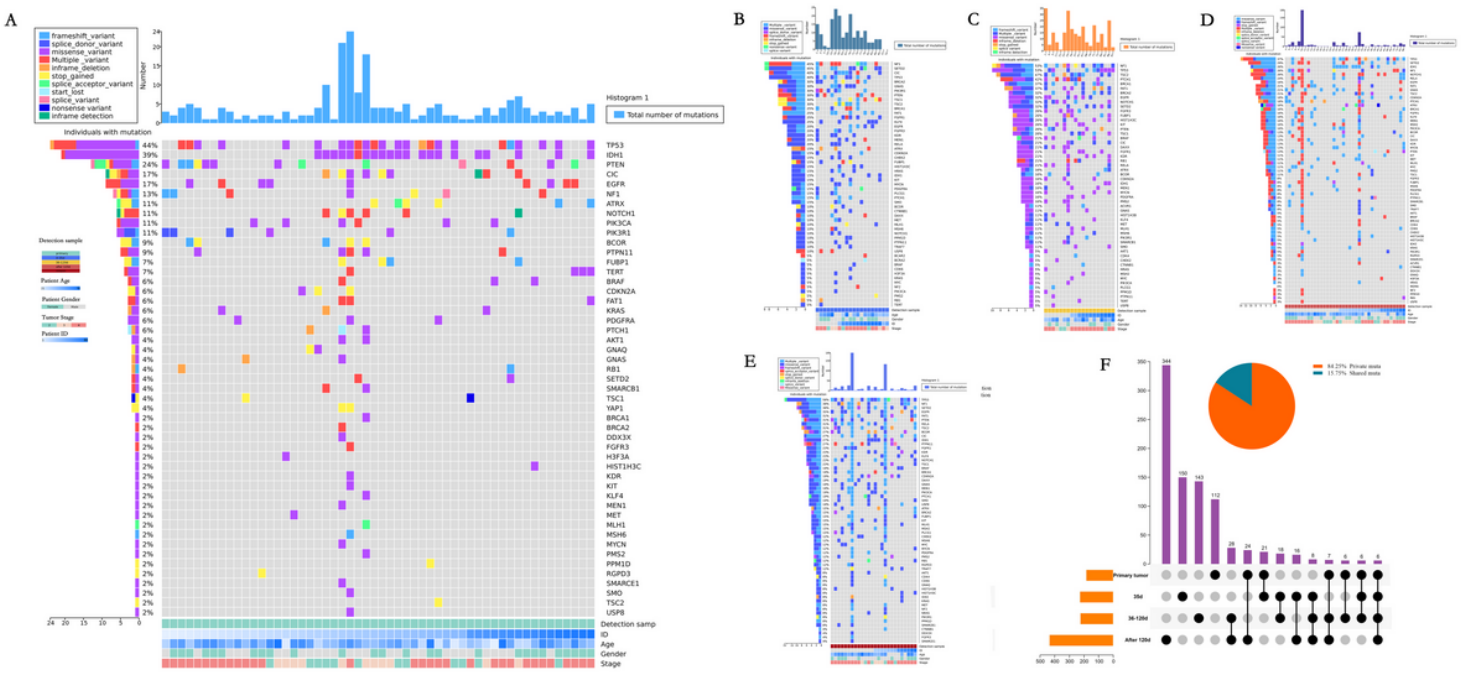

Figure 1

Genomic profiling of mutations between primary tissue samples and TISF samples. A-E. They were primary tumor tissue samples, TISF samples within 35 days after surgery, TISF samples 36-120 days after surgery, TISF samples 120 days after surgery, and TISF samples of recurrent tumors, including missense variant, Inframe deletion, and deletion. Frameshift variant, splice acceptor variant, stop gained, Multiple variant, frameshift variant, splice donor variant, start lost, splice variant, nonsense variant. **F.** The characteristics of shared mutation and private mutation in glioma map loci at different stages.

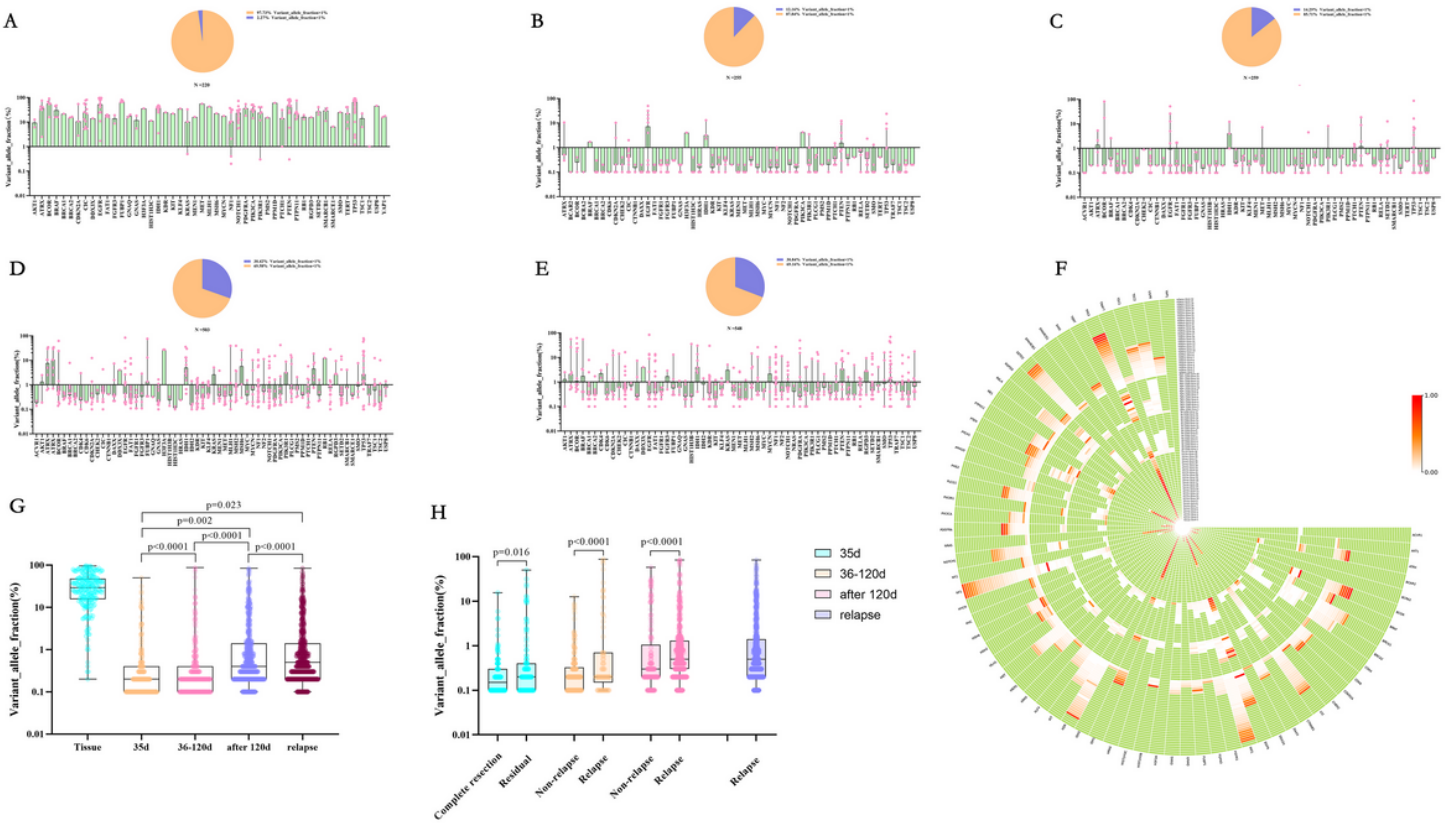

Figure 2

Allele variation frequency in the genome at different stages of first-line treatment for glioma. A-E. They were primary tumor tissue samples, TISF samples within 35 days after surgery, TISF samples 36-120 days after surgery, TISF samples 120 days after surgery, and TISF samples of recurrent tumors, different mutated genes and their Allele variation frequency, and low frequency VAF (<1%) ratios are shown. **F.** Allele Variation frequency of genes in all periods is integrated. High heat represents high VAF, the closer it is to the periphery, the closer it is to the recurrence time, and the center represents the primary tumor sample. **G.** Allele Variation frequency of mutated genes in different periods increased with tumor evolution, showing significant statistical differences. **H.** Allele Variation frequency in TISF samples of patients with postoperative imaging residue compared with patients with complete imaging resection and patients with recurrence compared with patients without recurrence was significantly different.

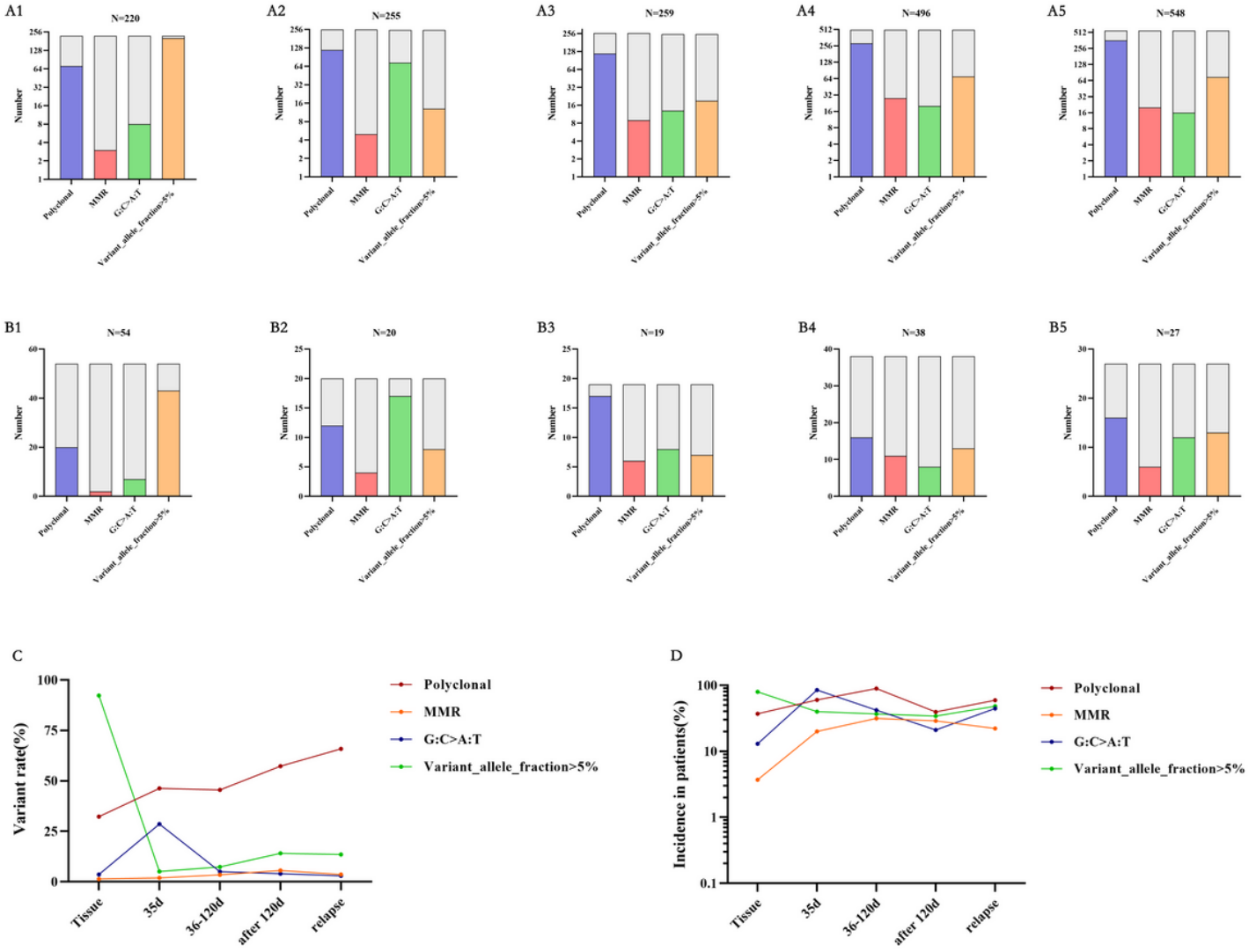

Figure 3

Changes in specific gene types in glioma under first-line treatment. A-D. The statistical categories of A, C and B, D are mutation sites and patients. (1-5) They were primary tumor tissue samples, TISF samples within 35 days after surgery, TISF samples 36-120 days after surgery, TISF samples 120 days after surgery, and TISF samples of recurrent tumors, include polyclonal mutations, MMR-related mutations of mismatch repair genes The accumulation of G:C>A:T and VAF greater than 5% of the genome changes.

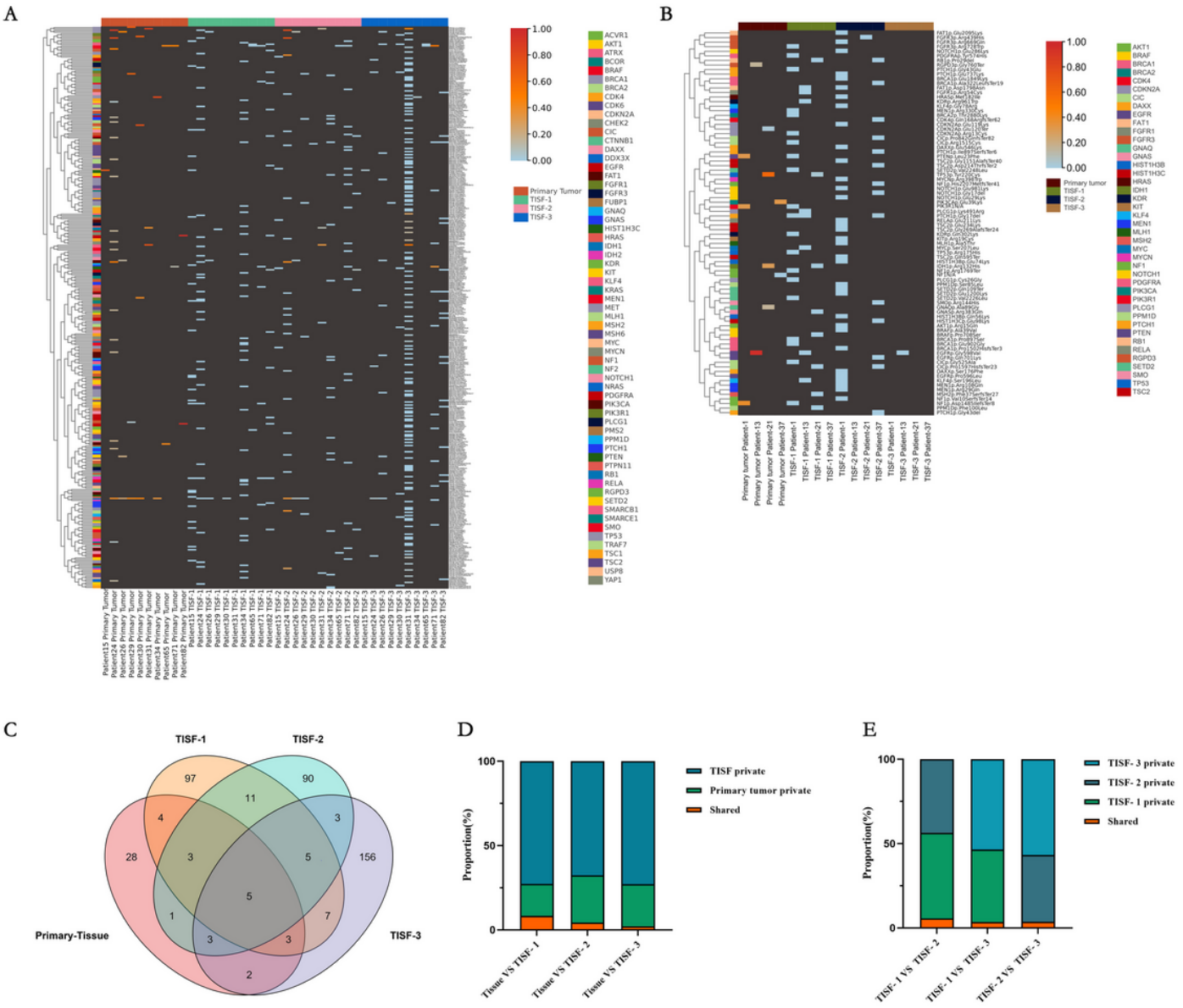

Figure 4

Genomic characteristics from patients who were continuously examined. A. Genomic profiling of mutations from 10 patients with relapses at follow-up. **B.** Genomic profiling of mutations in 4 patients who were followed without recurrence. **C.** In the process of continuous detection, the difference of mutation sites in different periods. **D-E.** The proportion of shared mutations and private mutations in different periods.

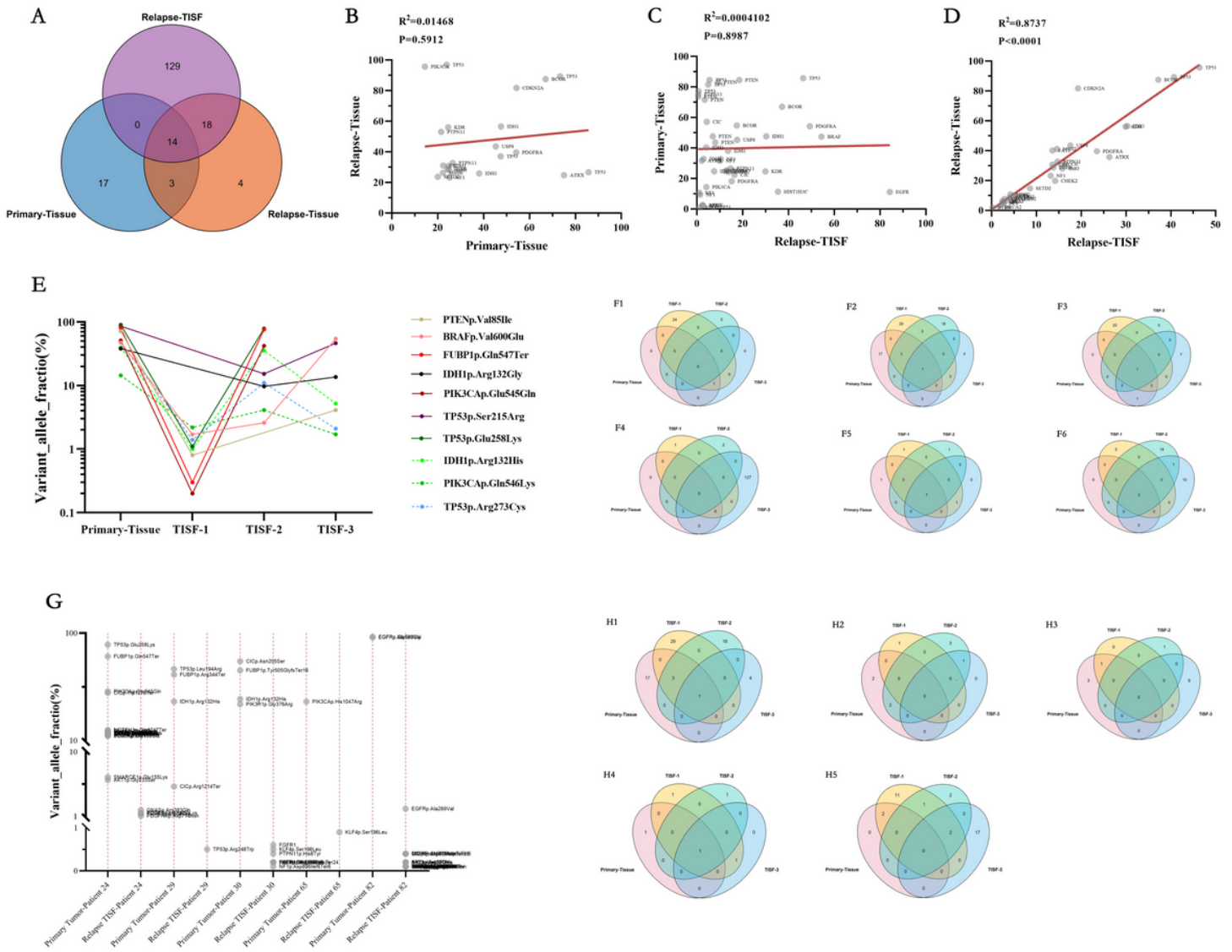

Figure 5

Genomic heterogeneity between recurrent and primary tumors. A. There were many mutated sites in the TISF of recurrent tumors, including most of the recurrent tissues (81.8%) and only 8.6% identical to the primary tumor tissue. **B-D.** TISF samples from recurrent tumors were consistent with tissue samples from recurrent tumors. **E.** F1-F6, dominant clonal mutation changes in 6 patients, with 7 gene loci up-regulated and 3 gene loci down-regulated. **G.** H1-H5, differential expression of private mutations in recurrent and primary genomes in 5 patients.

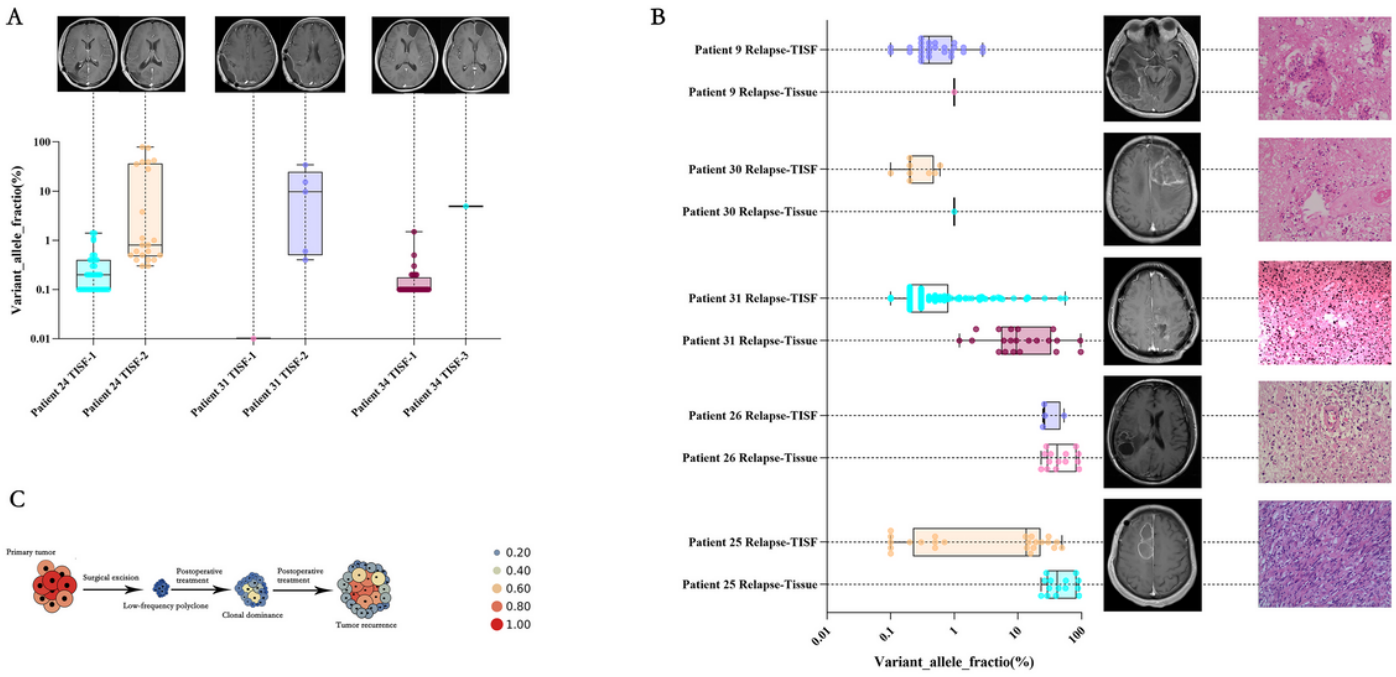

Figure 6

TISF samples were significantly correlated with clinical outcomes. A. Allele variation frequency increase in TISF was found in 3 patients with glioma after surgery before positive imaging examination, which may indicate recurrence of tumor DNA level. **B.** Five patients underwent a second operation, and pathology confirmed that TISF sample sequencing was more useful for clinical guidance than imaging results. **C.** Glioma evolution model under first-line treatment pressure, size and heat represent gene expression.