Hypermutagenesis in untreated adult gliomas due to inherited mismatch mutations

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Introduction

DNA repair mechanism is part of the essential cell homeostasis, ensuring genome integrity.1 Alterations in the DNA repair pathways, both hereditary and somatic, largely contribute to carcinogenesis and hypermutagenesis,2–4 manifesting specific genomic profiles and sensitivities to diverse therapies, including immunotherapy.5–11 As the number of systematic sequencing efforts have accumulated exponentially in recent years,12–14 prevalence of hypermutator genotype have become evident across multiple cancer types, including gliomas.1,15,16 Glioma is the most common primary malignant brain tumor in adults.17 The current

Key words: hypermutation, temozolomide, neoantigenicity, glioma, mismatch repair deficiency

Additional Supporting Information may be found in the online version of this article.

J.K.S. and S.W.C. contributed equally to this work.

Conflict of interest: The authors declare no competing financial interests.

Grant sponsor: Korea Health Technology R&D project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea; Grant number: HI14C3418

DOI: 10.1002/ijc.32054

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History: Received 3 Sep 2018; Accepted 29 Nov 2018; Online 11 Dec 2018

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Int. J. Cancer: 144, 3023–3030 (2019) © 2018 The Authors. International Journal of Cancer published by John Wiley & Sons Ltd on behalf of UICC
standard regimen for glioma patients consists of surgical resection followed by radiotherapy and/or concomitant and adjuvant temozolomide (TMZ) treatment.\textsuperscript{18,19} TMZ has been associated with improved prognosis in overall survival of the patients, specifically those with transcriptional silencing of the O-6-Methylguanine-DNA Methyltransferase (MGMT) gene, mediated by promoter methylation. However, previous literature has shown that a fraction of TMZ-treated patients acquired a hypermutator phenotype\textsuperscript{15,16,20} a direct result of TMZ-induced mutagenesis due to inactivation of the DNA mismatch repair (MMR) pathway, and they subsequently acquired treatment-induced therapeutic resistance.\textsuperscript{21–24} In our study, we report a rare subset of adult glioma patients with de novo hypermutator phenotype with no prior therapeutic intervention. We investigate whether TMZ-naive hypermutagenesis manifests distinct genomic or clinical features that present secondary mutagenic trajectory, suggesting potential alternative avenue for malignant transformation.

Materials and Methods

Clinical manifestations and glioma specimens

With appropriate approval from the institutional review board, all glioma specimens were obtained from patients undergoing surgery at the Samsung Medical Center. The study protocol was approved by our institution’s ethical committees, and written informed consents were received from all patients. After thorough analysis by pathologists, tumor specimens were snap-frozen and preserved in liquid nitrogen for genomic analysis.

Whole-exome sequencing

An Agilent SureSelect kit was used to capture exonic DNA fragments. An Illumina HiSeq 2000 instrument was used for sequencing and generating 2 x 101-bp paired-end reads.

Somatic mutation

The sequenced reads from the FASTQ files were aligned to the human genome assembly (hg19) using the Burrows-Wheeler Aligner (BWA) version 0.6.2. The initial alignment BAM files were subjected to conventional preprocessing before mutation calling: sorting, removing duplicated reads, locally realigning reads around potential small indels and recalibrating base quality scores using SAMTools, Picard version 1.73 and Genome Analysis ToolKit (GATK) version 2.5.2. We used Mutect (version 1.1.4) and Somatic IndelDetector (GATK version 2.2) to distinguish high-confidence predictions on somatic mutations between neoplastic and non-neoplastic tissue pairs. Variant Effect Predictor (VEP) was used to annotate the acquired somatic mutations.

Copy number alteration

ngCGH python package was used to generate estimated copy number alterations. The patient-matched normal WES data were used as reference to distinguish copy number changes in tumor specimens. The gene was labeled as “amplified” when the copy number was 3 or higher and “deleted” when it was 1.5 or lower.

Bulk RNA sequencing

RNA-seq libraries were prepared using the Illumina TruSeq RNA Sample Prep kit. Sequenced reads were subjected to quality trimming and mapped onto hg19 using GSNAP, not allowing mismatch, indels, or splicing. The resulting alignments were sorted and summarized into BED files using SAMTools and bedTools. The BED files were used to calculate values of RPKM (reads per kilobase of transcript per million reads) for each gene, using DEGseq package.

Isolation of single cells for RNA sequencing and single-cell analysis

We used the CITM Single-Cell Auto Prep System (Fluidigm) with the SMARTer kit (Clontech) to generate cDNAs. Cells were captured as a single isolate on a C1 chip, determined from bright-field images under 100x magnification using the Axiovert200 inverted microscope (Carl Zeiss). Libraries were generated using the NexteraXT DNA Sample Prep Kit (Illumina) and sequenced on the HiSeq 2,500 using 100-bp paired-end mode of the TruSeq Rapid PE Cluster kit and the TrueSeq Rapid SBS kit. Subsequent RNA reads were filtered at Q33 using Trimomatic-0.30 and mapped to reference. For single-cell analysis, we filtered out non-neoplastic cells based on transcriptome profiles. We employed the expression signatures of normal astrocytes, oligodendrocytes, neurons, microglia and other immune cells to classify individual cells according to their expression profiles. We further validated our annotation of malignant cells from non-malignant cells using the CONICSmat package to infer large-scale copy number variations (CNVs) from single-cell RNA-seq data.
Glioma-intrinsic transcriptional subtype analysis
To estimate glioma-intrinsic transcriptional subtype, we evaluated single sample Gene Set Enrichment Analysis (ssGSEA) for each subtype markers. Each corresponding subtype scores were normalized and the subtype with the highest z-score within each sample was assigned as its dominant subtype identity.

Results

HLA typing
HLA typing for each sample was performed using POLYSOLVER algorithm.25

Somatic mutation immunogenicity predictions
We used the pVAC-Seq pipeline with the NetMHCcons binding strength predictor to identify neoantigens.26 NetMHCcons integrates three state-of-the-art methods NetMHC, NetMHCpan and PickPocket to give the most accurate predictions.26 As required, we used the variant effect predictor from Ensembl to annotate variants for downstream processing by pVAC-Seq.27 For each single-residue missense alteration, HLA typing for each sample was performed using POLYSOLVER algorithm.25

MGMT promoter methylation
DNA was extracted from tumor tissue specimens using the QIAamp DNA Mini Kit (Qiagen) and the resulting DNA was modified with sodium bisulfite treatment using the EpiTect Bisulfite Kit (Qiagen). Afterward, the CpG island region of the MGMT was amplified and assessed using methylation/unmethylation specific primers. Methylation: TTTCCAGCCTT CGTAGGTTTTTCCG (forward) and GCACCTTCCTCGAAAAAC GAAAC (reverse). Unmethylation: TTTGTTGTTGTTGAT TTTGTTAGTTTTGT (forward) and AACTCCACACTTTC CCAAAAACAAAAA (reverse).28,29 Additionally, the reactions were validated by electrophoresis to verify the methylation status of the MGMT promoter. PCR products were 81 bp (methylation) and 93 bp (unmethylation), respectively.

Results

Genomic characterization of TMZ-naïve and TMZ-treated hypermutated tumors
To assess distinct genomic characteristics of TMZ-naïve hypermutated gliomas, we identified 14 patients (7 TMZ-naive and 7 TMZ-treated) with a hypermutator phenotype among 243 patients whose Whole-Exome Sequencing (WES) of both tumor specimens and matched normal blood were available (Table 1 and Fig. 1a). All hypermutated tumors exhibited extremely large numbers of non-synonymous somatic mutations with an average of 98.2 mutations/Mb, significantly exceeding the average number of mutation rates found in non-hypermutated tumors (Fig. 1a). Patients with post-TMZ-treated hypermutated tumors have undergone 6 to 18 cycles of temozolomide treatment. Both TMZ-naive and post-TMZ-treated cases showed somatic alterations in the core oncogenic pathways that are frequently dysregulated in glioblastoma including p53, Rb and receptor tyrosine kinase (RTK)/Ras/Phosphoinositide 3-kinase (PI3K) signaling pathways. Furthermore, glioma-intrinsic transcriptional subtype analysis revealed high prevalence of proneural cellular state in both groups (Fig. 1b). Consistent with prior notions that IDH1-mutated tumors are more susceptible to acquiring a hypermutator phenotype,15,20 majority of the TMZ-treated tumors were marked by somatic mutations in IDH1, while all TMZ-naive tumors demonstrated absence of IDH1 mutation (P = 0.02, Fisher’s exact test) (Fig. 1b).

Previous studies have identified a subset of GBM tumors with characteristic DNA methylation profile, commonly known as glioma CpG island methylator phenotype (G-CIMP).30,31 The global methylator phenotype is often induced by the gain-of-function IDH1 mutation at arginine 132 and the resulting tumors frequently demonstrate molecular and clinical features that are distinct from Non-G-CIMP tumors. The hypermethylation of the genome in G-CIMP tumors often encompass the promoter region of the O6-methylguanine-DNA methyltransferase (MGMT) gene. Subsequently, pMGMT methylation attributes to impairment of DNA repair mechanism32 and development of a hypermutator phenotype. Consistent with the previous reports, majority of the post-TMZ-treated hypermutated tumors demonstrated aberrant DNA methylation of the pMGMT (Fig. 1b) followed by attenuated mRNA expression level (Supporting Information Fig. S1). Conversely, TMZ-naive tumors mainly exhibited unmethylated pMGMT status.

Table 1. Clinical information of patients who were diagnosed with either treatment-naïve or treatment-received hypermutated gliomas

| Patient | Sex | Age | Pathology | Prior Tx |
|---------|-----|-----|-----------|---------|
| BT101†  | Male| 68  | GBM       | None    |
| BT102†  | Female | 40  | GBM       | None    |
| BT103   | Male | 45  | AA        | None    |
| BT104†  | Female | 21  | GBM       | None    |
| BT105   | Female | 34  | GBM       | None    |
| BT106†  | Female | 72  | GBM       | None    |
| BT107†  | Female | 63  | GBM       | None    |
| BT108   | Female | 56  | AODG      | RT + TMZ#12 |
| BT109   | Female | 56  | GBM       | RT + TMZ#6 |
| BT110   | Male  | 57  | AA        | RT + TMZ#18 |
| BT111   | Female | 53  | AODG      | RT + TMZ#9 |
| BT112   | Male  | 45  | GBM       | RT + TMZ#17 |
| BT113   | Female | 59  | GBM       | CCR+TMZ#6 |
| BT114   | Male  | 24  | GBM       | CCR+TMZ#6 |

AODG: Anaplastic Oligodendroglioma; GBM: Glioblastoma; AA: Anaplastic Astrocytoma; RT: radiotherapy; CCRT: concurrent chemoradiation therapy; TMZ: temozolomide.

†Patients had family history of malignant tumors.
Collectively, our results suggest that TMZ-naïve hypermutated tumors follow an alternative evolutionary path in acquiring a hypermutator phenotype.

**Dysregulation of mismatch repair (MMR) machinery**

DNA mismatch repair mechanisms are composed of various essentials, necessary for maintaining genome integrity and faithful replications. Mismatch repair (MMR) safeguards genome integrity through correcting improper nucleotide pairings that arise from replication errors. Dysfunction of the MMR largely contributes to accumulation of spontaneous mutations during tumor progression. Both TMZ-naïve and TMZ-treated tumors acquired somatic mutation of the MMR encoding genes including MSH (MSH2, MSH3, MSH5, MSH6; 12/14 tumors), MLH (MLH1, MLH3; 7/14 tumors) and/or PMS family (PMS1, PMS2; 5/14 tumors) (Fig. 2a). We also
Dysregulation of mismatch repair (MMR) encoding genes. 

Alterations in the MMR genes, is associated with increased risk of carcinogenesis including colon and endometrial cancers.33

As these genetic conditions are often hereditary, we expanded clinical assessment of the patients with germline MMR variations to their immediate family members. As suspected, several members of the patients’ families were previously diagnosed with cancer development history including brain tumor, lung, breast and prostate carcinomas. Consistently, several germline mutations have been formerly associated with cancer predisposing syndromes including rs63749919, \( \text{MSH6} \), \( \text{MSH3} \), \( \text{MSH6} \) and \( \text{MLH3} \). Lynch syndrome, an autosomal dominant genetic condition that is characterized by germline alterations in the MMR genes, is associated with increased risk of carcinogenesis including colon and endometrial cancers.33–35

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To further interrogate the clonality of TMZ-naïve hypermutated tumors at a single cell level, we curated single-cell sequencing data from multi-region tumor samples (main mass and resection margin) from an individual patient (BT104). To distinguish non-malignant from malignant cells, we estimated inferred copy-number variations for individual cells using gene expression profiles over large chromosomal regions within each cell. Each tumor cell demonstrated large-scale chromosomal aberrations, including amplification of chromosome 8 and deletions of 5,10 and 13 (Supporting Information Fig. S2). On the contrary, cells that were classified as non-malignant cells lacked any detectable CNVs. Consistent with the bulk WES results, mutations of the MMR encoding genes including, \( \text{PMS1} \), \( \text{MLH3} \) and \( \text{MSH3} \), were detected across all single cells with enough coverage at the corresponding genomic regions (Fig. 2c).

Additionally, we also discovered a high frequency of \( \text{PDGFR} \) mutations (60%, 83/138), which significantly associated with a proneural expression phenotype. These observations were consistent with the bulk WTS results, where proneural transcriptional subtype was predominant among hypermutated tumors.

**Distinct mutational signatures and elevated neoantigen loads in hypermutated tumors**

Next, we evaluated mutational signatures of TMZ-naïve and TMZ-treated hypermutated tumors.36,37 Of the six classes of base substitution in the mutation type, both groups exhibited robust presence of C-to-T transition (Fig. 3a). TMZ-naïve tumors were characterized by predominance of C-to-T substitutions at NpCpG trinucleotides (where N represents any nucleotide), which has been speculated to be a direct result of elevated rate in spontaneous deamination of 5-methylcytosine38 (Fig. 3b). On the other hand, post-TMZ treatment tumors were enriched with excess C-to-T substitutions at NpCpC and NpCpT trinucleotides, which is an evident indication of TMZ-induced mutagenesis.36,37 (Fig. 3b). Notably, majority of the driver mutations that were observed in the post-TMZ treatment tumors consist
of C-to-T substitutions at NpCpC and NpCpT contexts, suggesting that these tumors have undergone TMZ-induced malignant progressions.

High mutational burden has been shown as an integral indication of neoantigen production and presents potential immunotherapeutic intervention across multiple cancer types.6,8,9,39
Therefore, we have assessed neoantigenic levels in both TMZ-naïve and post-TMZ-treated hypermutated tumors compared to non-hypermutated tumors. Notably, both hypermutated tumors exhibited a significant accumulation of neoantigen load (Fig. 3c), suggesting potential immunotherapeutic strategy for these patients.

**Discussion**

In our study, we report for the first time a group treatment-naïve adult glioma patients with a hypermutator phenotype and explore their spontaneous genomic profiles that constitute an alternative mutagenic process. Notably, TMZ-naïve hypermutated tumors were primarily marked by absence of IDH1 somatic mutation and pMGMT methylation, two genomic features that were significantly associated with development of hypermutation in GBM.16 Transcriptional silencing of MGMT gene via promoter methylation, followed by dysfunction of the MMR encoding genes were presumably the underlying mechanism behind TMZ-induced hypermutator phenotype.12,40,41 Furthermore, hypermutagenesis in glioma has been exclusive to recurrent tumors as it has been perceived as a direct result of TMZ intervention that subsequently lead to therapeutic resistance to standard treatment regimen. Therefore, it remained controversial whether inactivation of the MMR encoding genes or hypermutation was directly associated with acquisition of TMZ-induced resistance. Our results have shown that spontaneous de novo hypermutated tumors follow an alternative evolutionary trajectory in acquiring continuous mutagenic process via combination of germline and somatic mutations of the mismatch repair genes. Despite having such features, patients with de novo hypermutation exhibited favorable clinical responses to the standard TMZ chemotherapy (Supporting Information Fig. S3), in contrast to the previous notion that inactivation of MSH6 could potentially confer TMZ resistance in glioblastoma.24 Although recent literatures have identified multiple brain tumor types to be associated with Lynch syndrome MMR mutational carriers, including MLH1 and MSH2,42–44 TMZ-naïve patients primarily demonstrated high prevalence MSH3 and MSH6 germline variations. Our results suggest that inherited dysfunction of the MMR pathway through MSH3 or MSH6 mutation could potentially pose hereditary risk to genetic predisposition of carcinogenesis. Our speculations were further corroborated via expanded evaluation of the patients’ clinical history. Notably, patients with TMZ-naïve hypermutator phenotype demonstrated a significantly higher incidence of cancer-related occurrences in their immediate family members (Supporting Information Table S1). These observations suggest that inherited mutations in the MMR encoding genes could potentially promote tumor initiation and further impairment of the DNA repair mechanism via newly acquired somatic variation could result in hypermutation development.

A substantial body of evidence has highlighted significant association between patients with elevated neoantigen load and improved clinical response to checkpoint-blockade immunotherapy across various cancer types including glioblastoma.6–9,39 Interestingly, our results showed increased neoantigen counts in both TMZ-naïve and post-TMZ-treated hypermutated tumors, suggesting potential implication of checkpoint inhibitors in these patients. Even though hypermutagenesis in a newly-diagnosed glioma is a rare event, it is important that all patients should be evaluated for a hypermutated genotype as early identification of glioma patients prior to temozolomide treatment could potentially lead to new clinical trials in evaluating replacement of alkylating agents with checkpoint inhibitors. However, further studies are warranted in implementing immunotherapeutic approach in glioma patients as CNS malignancies are often void of cytotoxic immune cells such as T cells, which are critical components in immune checkpoint inhibition. Therefore, only a subset of glioma patients could be appropriate targets for immunotherapeutic approach in defined scenarios, such as presence of a hypermutator phenotype. As such, our results provide a conceptual groundwork for clinical practice against glioma patients with de novo hypermutation.

Although complete understanding of the underlying mechanism behind spontaneous hypermutagenesis in glioma requires an in-depth experimental validation, we demonstrate that combined inherent and somatic mutations of the mismatch repair encoding genes could largely contribute to development of de novo hypermutator phenotype in gliomas. Furthermore, our results present an important step towards potential implementation of immunotherapy in glioma treatment.

**Acknowledgements**

The biospecimens for our study were provided by Samsung Medical Center BioBank.

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