Molecular Landscape for Malignant Transformation in Diffuse Astrocytoma

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Abstract

Background Malignant transformation (MT) of low-grade gliomas changes dramatically the natural history to poor prognosis. Currently, factors associated with MT of gliomas have been inconclusive, in particular, diffuse astrocytoma (DA).

Objective The present study aimed to explore the molecular abnormalities related to MT in the same patients with different MT stages.

Methods Twelve specimens from five DA patients with MT were genotyped using next-generation sequencing (NGS) to identify somatic variants in different stages of MT. We used cross-tabulated categorical biological variables and compared the mean of continuous variables to assess for association with MT.

Results Ten samples succussed to perform NGS from one male and four females, with ages ranging from 28 to 58 years. The extent of resection was commonly a partial resection following postoperative temozolomide with radiotherapy in 25% of cases. For molecular findings, poly-T-nucleotide insertion in isocitrate dehydrogenase 1 (IDH1) was significantly related to MT as a dose–response relationship (Mann–Whitney’s U test, p = 0.02). Also, mutations of KMT2C and GGT1 were frequently found in the present cohort, but those did not significantly differ between the two groups using Fisher’s exact test.

Conclusion In summary, we identified a novel relationship between poly-T insertion polymorphisms that established the pathogenesis of MT in DA. A further study should be performed to confirm the molecular alteration with more patients.

Introduction

Malignant transformation (MT) of low-grade gliomas (LGGs), which is the progression of benign tumor cells converting to malignancy, was found in 19.5 to 21%.1–4 The 10-year cumulative incidence of MT was 3.8% and the median time of MT was 5.1 years from a study of Broniscer et al.,1 while other prior studies reported MT rates in LGGs in 19.5%.2 Additionally, the MT of these benign tumors changed the natural history of the disease and also accelerated to a poor prognosis.4–6 Currently, the pathogenesis of MT is still being debated. Murphy et al studied 599 patients with LGG and found the...
incidence of MT was 21%. From the results of prior studies, risk factors associated with MT were older age, male gender, multiple tumors, chemotherapy alone, and the extent of resection were potential predictors of MT.6 However, radiation exposure was reported as a significant factor associated with MT in the study of Sakarunchai et al.2 Furthermore, molecular alterations had an impact on MT in various previous studies. TP53 overexpression, deletions of RB1, Cdkn2A, and PTEN pathway abnormalities were related to MT.1,4 Based on the previous study, molecular alterations were significantly associated with MT.6

As MT in LGG, especially diffuse astrocytoma (DA), is not a common event, the transformation processes need time and long-term follow-up. Hence, a lack of evidence of MT in the specific group of DA comprised benign astrocyte cells directly turning to malignant cells from the literature review. The heterogeneity in various types of LGG such as DA, oligodendroglioma might interfere with the result of genetic alterations related to MT.2,3 Moreover, a comparison of genetic alterations in the same patient has been rarely reported. Hence, we conducted a molecular study of DA exploring the hypothesis of the pathway of MT in high-grade astrocytoma (HGA). The present study aimed to compare the molecular alterations between DA and HGA in the same individual using the next-generation sequencing (NGS).

Materials and Methods

Study Population

Patients who were diagnosed with DA and HGA in the same patient at different times between 2016 and 2020 were included. The study was performed with the approval of the Human Research Ethics Committee of Faculty of Medicine, XXX (REC 61-372-10-1). The informed consents were obtained and signed routinely from all patients before tumor specimen collection at the biobank of the Faculty of Medicine, Prince of Songkla University.

Frozen Tissue Specimens

Tumor specimens were collected at the time of craniotomy operation and frozen at –40°C until use. Samples were histologically reviewed by a pathologist to confirm the diagnosis at each stage of MT. Using a High Pure PCR Template Preparation Kit (Roche, Berlin, Germany), deoxyribonucleic acid (DNA) was extracted from the frozen tumor specimens. DNA extraction was performed according to the manufacturer’s instructions as per the previous study.7,8

Next-Generation Sequencing and Data Processing

Following basic quality control assessment, the DNA libraries were sequenced on an Illumina NovaSeq 6000 platform with 150 pair-end read format with an average depth of 40x (median = 30x). The raw sequencing reads were achieved using FastQC; therefore, trimming and mapping to the human genome (GRCh38, UCSC hg38) using the Burrows-Wheeler Aligner (BWA) software program.3 Using the Genome Analysis Toolkit (GATK), the indel realignment over the overlapping target regions was performed.10 Hence, SnpEff was used to do the variant calling and identify single nucleotide polymorphism (SNP), and insertion and deletion were annotated.

Statistical Analysis

The association between gene mutation and clinical data was analyzed using the chi-square test and/or Fisher’s exact tests. Hence, the Mann–Whitney’s U test was used to compare the mean between the two groups. The statistical tests were two sided, and a value of \( p < 0.05 \) was considered statistically significant. Moreover, clinical characteristics and magnetic resonance imaging (MRI) of the brain were collected from medical records and databases for clinical correlation propose. The waterfall plots were performed to demonstrate mutation landscape using R version 4.0.4 with the “GenVisR” package.11,12 Moreover, the MRIs of the brain were integrated into the waterfall plots for visualization.

Results

Patient Demographics and Clinical Characteristics

Twelve tumor specimens from five patients were included in the present study and the male-to-female ratio was 0.20:1. The median age at diagnosis was 45 years (interquartile range: 10) and all tumors involved the frontal lobe. The mean follow-up duration for the present cohort was 50 months and the baseline clinical characteristics and outcomes are revealed in –Table 1. The extent of resection was that the majorities were partial resection following adjuvant treatment. Twenty-five per cent of the present cohort received temozolomide when DA transformed to glioblastoma because the high cost of temozolomide is the major cause that temozolomide has not been implemented as the standard treatment in health resource-limited settings. Hence, DA patients had a median MT-free time of 7 months (95% confidence interval [CI]: 0.14–14.63) and had a poor prognosis with a median survival time of 17 months (95% CI: 14.85–19.14). Moreover, we then analyzed the associations between the presence of MT and baseline characteristics of patients. As a result, the clinical characteristics did not significantly associate with MT of DA. Following DNA extraction, nine samples passed quality check control with an average quality check control for NGS.

Mutational Landscape in DA with MT

Genetic alterations which were frequently found among samples were IDH1 (rs34363027, rs386392441, rs71412484, rs1446325, rs7383668, rs796498057), IDH2 (rs60147683, rs2970357), GGT1 (rs768399767), and KMT2C (rs58528565), as shown in –Table 2. IDH1 mutation was present in all specimens, but we did not find the IDH1 R132H hotspot mutation in our cohort. However, we observed the dose–response relationship of insertion of T-nucleotides and MT of DA. CT/CTT polymorphism was found in the pre-MT stage of DA, while higher poly-T insertion (CTT/CTTTT) polymorphism was found in HGA. Moreover, HGA had a significantly higher mean number of T insertion than DA (Mann–Whitney’s U test,
p = 0.02), while other SNPs did not significantly differ between the two groups using Fisher’s exact test.

IDH2 mutation is one of the genes associated with LGG in the literature review. In the present study, we found the IDH2 R172K hotspot mutation in 33.3% (3/9) of all specimens. GGT1 and KMT2C mutations were frequently found in our cohort at 55.5% (5/9) and 66.6% (6/9), respectively. However, other SNPs did not significantly differ between the two groups using Fisher’s exact test.

Table 1 Demographic data of astrocytoma patients with malignant transformation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Histopathology</th>
<th>Time to MT, mo</th>
<th>Extent of resection</th>
<th>Adjuvant therapy</th>
<th>Survival time, mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1 (48-y-old woman)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>Diffuse astrocytoma at the left frontal lobe</td>
<td></td>
<td>Partial resection (70%)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>Anaplastic astrocytoma</td>
<td>1 mo after first operation</td>
<td>Subtotal resection (80%)</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>Glioblastoma</td>
<td>4 mo after first operation</td>
<td>Subtotal resection (80%)</td>
<td>Re-RT with TMZa</td>
<td>Death at 23 mo after first operation</td>
</tr>
<tr>
<td>Patient 2 (45-y-old man)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>Diffuse astrocytoma at the left frontal lobe</td>
<td></td>
<td>Subtotal resection (80%)</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td>Glioblastoma</td>
<td>44 mo after first operation</td>
<td>Total resection</td>
<td>Re-RT with TMZ</td>
<td>Alive (50 mo after first operation)</td>
</tr>
<tr>
<td>Patient 3 (28-y-old woman)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>Diffuse astrocytoma at the right frontal lobe</td>
<td></td>
<td>Partial resection (70%)</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>T7†</td>
<td>Anaplastic astrocytoma</td>
<td>7 mo after first operation</td>
<td>Subtotal resection (90%)</td>
<td>Adjuvant vincristine/cyclophosphamide</td>
<td></td>
</tr>
<tr>
<td>T8</td>
<td>Glioblastoma</td>
<td>14 mo after first operation</td>
<td>Partial resection (70%)</td>
<td>Best supportive care</td>
<td>Death at 16 mo after first operation</td>
</tr>
<tr>
<td>Patient 4 (58-y-old woman)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T9b</td>
<td>Diffuse astrocytoma at right parasagittal area</td>
<td></td>
<td>Neuronavigation-guided biopsy</td>
<td>RT</td>
<td></td>
</tr>
<tr>
<td>T10b</td>
<td>Glioblastoma</td>
<td>15 mo after first operation</td>
<td>Subtotal resection (80%)</td>
<td>Re-RT</td>
<td>Death 17 mo after first operation</td>
</tr>
<tr>
<td>Patient 5 (32-y-old woman)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T11</td>
<td>Diffuse astrocytoma at the right frontal lobe</td>
<td></td>
<td>Partial resection (70%)</td>
<td>–</td>
<td>Death at 5 mo after first operation</td>
</tr>
<tr>
<td>T12</td>
<td>Glioblastoma</td>
<td>3 mo after first operation</td>
<td>Partial resection (70%)</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: MT, malignant transformation; RT, radiotherapy; TMZ, temozolomide.

†Progression of the residual tumor within 1 month following third operation and two reoperations were performed later (total operations were five).

bTumor specimens did not pass quality control before whole exome sequencing.

In detail, one patient collected tumor specimens in all stages of MT in the present cohort. PRKCQ, PPIF, NRPI, MEVF, GGT1, FAN1, and BTK mutations were found in both DA and anaplastic astrocytoma, whereas mutations of TBC1D19, ESRA, DIAPH2, COG6, and CBWD3 occurred in HGA. As shown in ►Fig. 1, various genetic mutations were found in each stage of MT. Also, ZC3H18, SLIT2, CARF, GPATCH4, CAMK2D, BCORL1, ARHGAP4 mutations were found in glioblastoma (T03).
The remaining patients also compared genetic alterations between DA and glioblastoma, as shown in Fig. 2. Glioblastoma (T05, T08, and T12) developed mutation as follows: USP22, TTN, SMC3, RFX4, RFX7, PREPL, PAN3, FAM58A, DSC1, DMD, COL4A5, NOXA1, HIP1R, FOLH1, DUOX2, CUL7, V8X2, TP53, SIK1, PIK3CA, KHSRP, CYP2A6, and ATG16L2. From the present cohort, roles of MT were hypothesized and established for confirmatory study in the future as shown in Fig. 3. Moreover, we identified other various somatic alterations in each sample based on the Catalogue of Somatic Mutations in Cancer (COSMIC) as a supplement.

**Discussion**

MT in DA is an uncommon feature and occurs in approximately 20% of all DA and needs time to develop a malignancy. In the present study, five patients with DA developed MT within 7 months, which was a shorter duration than prior studies that reported the median time of MT was 61 to 68 months in LGG. This is potentially explained by the heterogeneity of population studies that the present study focused specifically on DA, while prior studies included DA, oligodendroglioma, and oligoastrocytoma. However, this is in concordance to two previous studies that the prognosis of those with MT is poor. The comparison of genetic mutations among stages of MT may be a way to explore the pathophysiology of MT.

Following the 2016 WHO CNS tumor classification, the mutations of IDH1 and IDH2 are the key molecular alterations driving the pathogenesis of gliomas. The hotspot mutations of those genes were uncommonly found in the present study, but there was observed evidence of poly-T-nucleotide insertion correlated to MT among DA as the dose–response relationship

### Table 2 Genetic alterations of IDH1, IDH2, and other essential genes

<table>
<thead>
<tr>
<th>rs</th>
<th>Gene</th>
<th>Chrom</th>
<th>Ref</th>
<th>Alt</th>
<th>Type</th>
<th>Position</th>
<th>Effect</th>
<th>COSM ID</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1446325</td>
<td>IDH1</td>
<td>2</td>
<td>C</td>
<td>T</td>
<td>SNP</td>
<td>209120640</td>
<td>Upstream gene variant</td>
<td>–</td>
<td>T01, T02, T03, T04, T06, T08, T11, T12</td>
</tr>
<tr>
<td>rs57383668</td>
<td>IDH1</td>
<td>2</td>
<td>GA</td>
<td>G</td>
<td>DEL</td>
<td>209101905</td>
<td>Intron variant</td>
<td>–</td>
<td>T01, T02, T03, T04, T05, T06, T08, T11, T12</td>
</tr>
<tr>
<td>rs796498057</td>
<td>IDH1</td>
<td>2</td>
<td>C</td>
<td>T</td>
<td>SNP</td>
<td>209110270</td>
<td>Intron variant</td>
<td>–</td>
<td>T01</td>
</tr>
<tr>
<td>rs34363027</td>
<td>IDH1</td>
<td>2</td>
<td>C</td>
<td>T</td>
<td>SNP</td>
<td>209110270</td>
<td>Intron variant</td>
<td>–</td>
<td>T02</td>
</tr>
<tr>
<td>rs386392441</td>
<td>IDH1</td>
<td>2</td>
<td>C</td>
<td>T</td>
<td>SNP</td>
<td>209110270</td>
<td>Intron variant</td>
<td>–</td>
<td>T03</td>
</tr>
<tr>
<td>rs71412484</td>
<td>IDH1</td>
<td>2</td>
<td>C</td>
<td>T</td>
<td>SNP</td>
<td>209110270</td>
<td>Intron variant</td>
<td>–</td>
<td>T04</td>
</tr>
<tr>
<td>rs11540478</td>
<td>IDH1</td>
<td>15</td>
<td>G</td>
<td>A</td>
<td>SNP</td>
<td>90628537</td>
<td>Intron variant</td>
<td>–</td>
<td>T01, T02, T03</td>
</tr>
<tr>
<td>rs2970357</td>
<td>IDH2</td>
<td>15</td>
<td>T</td>
<td>C</td>
<td>SNP</td>
<td>90623052</td>
<td>Intronic variant/Downstream gene variant</td>
<td>COSM3754569</td>
<td>T01, T02, T03, T04, T05, T06, T08, T11, T12</td>
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<td>rs768399767</td>
<td>GGT1</td>
<td>22</td>
<td>G</td>
<td>A</td>
<td>SNP</td>
<td>25023537</td>
<td>Missense variant</td>
<td>COSM1032740</td>
<td>T01, T02, T05, T08, T11</td>
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<tr>
<td>rs58528565</td>
<td>KMT2C</td>
<td>7</td>
<td>G</td>
<td>C</td>
<td>SNP</td>
<td>151927023</td>
<td>Stop gained</td>
<td>COSM216053</td>
<td>T01, T02, T03, T05, T06, T08, T11</td>
</tr>
</tbody>
</table>

Abbreviations: Alt, alteration; Chrom, chromosome; DEL, deletion; INS, insertion; Type, transcription effect; rs, Ref; SNP, single nucleotide polymorphism.
of poly-T-nucleotide insertion. In detail, more T-nucleotide insertions were significantly observed in higher grade and severity of astrocytoma that may involve splicing errors during transcriptions. This finding is a novel mutation that is found in gliomas, the intronic poly (AT) deletion/insertion polymorphism of the XPC gene that has been found in urinary system cancer and breast cancer from literature review.\textsuperscript{16,17} Following 32 publications, Dai et al conducted a meta-analysis for identifying the association between this polymorphism and the risk of urinary system cancer in 10,214 cases and 11,302 controls. The results found that polymorphism has a significantly increased risk of urinary cancer.\textsuperscript{18} Moreover, this polymorphism has been reported as a risk factor for various cancers such as breast cancer,\textsuperscript{17} gastric cancer,\textsuperscript{19} and squamous cell carcinoma of the head and neck.\textsuperscript{20,21}

In addition, we found that KMT2C and GGT1 were common in the present cohort. These molecular alterations have been reported in various cancers, but there was a lack of evidence supporting these mutations in glioma. Cho et al studied the mutation of KMT2C in diffuse-type gastric adenocarcinoma and found that these promoted epithelial-to-mesenchymal transition and were associated with a short survival time.\textsuperscript{22} Gala et al studied KMT2C mutation in breast cancer and found that the deletion of KMT2C was associated with poor prognosis via hormone-driven estrogen receptor α activity.\textsuperscript{23} GGT1 transcribes gamma-glutamyltransferase 1 that is associated with a favorable prognosis in renal and ovarian cancers, while these were significantly associated with mortality in the metastatic pancreatic ductal adenocarcinoma.\textsuperscript{24,25} However, those need further exploration, the association with MT in DA, from more samples because there was the limitation of a small sample size. The process of MT occurred in the range of 7 to 68 months, and the routine collection of tumor specimens in the tumor bank will increase the number of specimens for further confirmatory study in the future.

Additionally, certain limitations should be acknowledged. The present study explored the genetic profiles at DNA level related to MT; therefore, a lack of comparison between the MT case and the non-MT control groups was observed. Also, studies of the transcriptome and protein expression challenge to confirm these intronic poly-T insertion polymorphisms. However, the present study is the first study to compare molecular profiles in the same patients with different MT stages that establishes the role of MT in DA.

For future research, further studies should be performed on NGS for comparison between MT cases and non-MT

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**Fig. 1** Mutation landscape of malignant transformation in each stage. Waterfall plot shows mutation landscape found of diffuse astrocytoma (T01), anaplastic astrocytoma (T02), and glioblastoma (T03) in the same patient. The type of mutation is represented by different colors. The bottom illustrates contrast-enhanced T1-weighted magnetic resonance imaging of the different stages.
Fig. 2 Comparison of mutation landscape between diffuse astrocytoma and glioblastoma. The waterfall plot shows mutation landscape which found diffuse astrocytoma (T04, T06, T12) and glioblastoma (T05, T08, T12) and black solid lines connect the same patient. The type of mutation is represented by different colors. The bottom illustrates contrast-enhanced T1-weighted magnetic resonance imaging of each individual at different stages.

Fig. 3 Hypothesized roles of malignant transformation in diffuse astrocytoma.
controls. Besides, the results of the present study need to be confirmed with molecular findings using more numbers of patients or further study performed in the archival specimens using direct DNA sequencing.

**Conclusion**

In summary, we identified a novel significant dose–response association between poly-T insertion polymorphisms that establishes the pathogenesis of MT in DA.

**Conflict of Interest**

None declared.

**References**

8. Tunthanathip T, Sangkhathat S. Temozolomide for patients with wild-type isocitrate dehydrogenase (IDH1) glioblastoma using propensity score matching. Clin Neurol Neurosurg 2020;191:105712