主論文の要旨

Next Generation Sequencing-Based Transcriptome Predicts Bevacizumab Efficacy in Combination with Temozolomide in Glioblastoma

（次世代シーケンシングに基づくトランスクリプトームは
膠芽腫におけるベバシズマブのテモゾロミドとの
組合せ効果を予測する）

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【Introduction】

Glioblastoma (GBM) is the most common and malignant brain tumor. The standard treatment is maximal resection, radiotherapy, and Temozolomide (TMZ, an alkylating agent). Compared with primary GBM which initially develops as GBM, secondary GBM mostly progresses from lower grade glioma (LGG), and the *isocitrate dehydrogenase (IDH)* mutation is predominantly observed in secondary GBM and LGG. However, it remains to be elucidated which genetic alterations are involved in the malignant transformation of LGGs into secondary GBM.

Due to the development of next generation sequencing (NGS) technologies, genetic analysis for GBM has progressed rapidly in recent years. RNA sequencing (RNA-seq) with NGS is a newly developed powerful method that enables the quantification of the expression level of genome-wide genes without previously prepared probes and the risk of non-specific hybridization.

In this study, we performed transcriptomic analysis by NGS and revealed that the inhibition of the vascular microenvironment played important roles in tumor growth in the *IDH1*-mutant GBM xenograft. As GBM is known to secrete vascular endothelial growth factor (VEGF) and actively induce angiogenesis, Bevacizumab (Bev; an anti-VEGF humanized monoclonal antibody) has recently been administered monotherapy to extend overall survival time (OS) in patients with recurrent GBM. For newly-diagnosed GBM, Bev in combination with standard treatment significantly prolonged progression free survival (PFS), but not OS. However, no large-scale clinical trials or comprehensive results on the combined use of Bev and TMZ have been reported with regard to the *IDH1*-mutant GBM; therefore, the effect of *IDH1* mutation on these therapies is unknown.

【Methods】

The human GBM cell line U87 was transduced to express endogenous wildtype *IDH1* or mutant *IDH1-R132H*. The *IDH1*-wildtype U87 and *IDH1*-mutant U87 cell lines were seeded at a density of 2500 cells/well in 96-well plates and 2.0×10^4 cells/well in 24-well plates. After cultured 24h with DMEM, cells were treated for 120h respectively with Bev (500 ng/mL), TMZ (97 μg/mL), the combination of Bev (500 ng/mL) and TMZ (97 μg/mL), and 0.5 % Dimethyl Sulfoxide (DMSO) for control group. The cell viability in 96-well plates was detected by cell counting kit-8 (CCK-8) assay. Total RNA was extracted from cells in 24-well plates and RNA with RNA Integrity Number (RIN) scores > 8.5 was used to prepare cDNA libraries for RNA-seq. NGS reads sequenced with the Illumina Hiseq 2500 were aligned to the hg19 genome + transcriptome assembly (UCSC hg19) using TopHat v2.1.1 with the default parameters. Read counts of each gene were obtained using HTSeq and differential expression was analyzed using DESeq. Gene Set Enrichment Analysis (GSEA) was performed using the GSEA JAVA program. Gene Ontology (GO)

analysis of genes whose expression level changed significantly ($p < 0.05$) by more than two-fold after adding Bev to TMZ in *IDH1*-mutant U87 cells was performed.

To prepare a subcutaneous tumor model, five-week-old nude mice were subcutaneously injected with 5×10^6 cells of *IDH1*-wildtype U87 or *IDH1*-mutant U87 cell line respectively. When the tumor diameter reached 5 mm, 10 mg/kg of Bev, 7.5 mg/kg of TMZ, a combination of Bev (10 mg/kg) and TMZ (7.5 mg/kg), or DMSO (control) were administered intraperitoneally. The same amount of TMZ was intraperitoneally administered for five consecutive days. The tumor volume of each treatment group was measured daily with the start of reagent administration. The cell viability and chemo-sensitivity data were analyzed by Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

【Results】

RNA-seq revealed highly different genome-wide expression profiles in *IDH1*-mutant U87 and *IDH1*-wildtype U87 cells treated with Bev + TMZ. GO suggested that Bev + TMZ upregulated genes for extracellular matrix organization and immune response, but downregulated genes for cell cycle progression in *IDH1*-mutant U87 cells (**Figure 1**). Similarly, GSEA suggested that the combination of Bev and TMZ activated genes related to immunocyte migration (myeloid leukocyte migration, leukocyte chemotaxis, and lymphocyte migration/ chemotaxis), but suppressed genes related to the cell cycle (DNA replication, mitotic recombination, chromosome condensation, and DNA strand elongation) in *IDH1*-mutant U87 cells, whereas there were no significant changes in *IDH1*-wildtype U87 cells (**Figure 2**).

CCK-8 assays showed that, there was no significant difference in the number of living cells in the Bev monotherapy group compared to the control group in either the *IDH1*-wildtype or *IDH1*-mutant U87 cells. Although TMZ showed a significant antitumor effect, the addition of Bev to TMZ showed no significant add-on effect in both cell lines (**Figure 3**).

In vivo studies, Bev + TMZ suppressed tumor growth only in the *IDH1*-mutant U87 xenograft, whereas neither TMZ nor Bev monotherapy inhibited tumor growth in either cell type and *IDH1*-wiltype tumors did not respond to Bev + TMZ (**Figure 4**).

【Discussion】

According to one of the largest reports of The Cancer Genome Atlas (TCGA), GBM with *IDH* mutation accounts for only a small percent of all gliomas. Consequently, there has only been a few reports on specific gene expression profiling of *IDH1*-mutant GBM in comparison with that of the *IDH1*-wildtype GBM, although the *IDH1* mutation in glioma has been found to cause extensive DNA hypermethylation leading to suppression of gene expression. *IDH1*-mutant glioma tends to display a lower expression of programmed death

ligand 1 (PD-L1), a smaller number of tumor-infiltrating lymphocytes (TILs), and a lower expression of genes related to $CD8^+$ cytotoxic T lymphocytes (CTL) and IFN-γ than those of the *IDH1*-wildtype counterparts.

About Bev monotherapy, there has been reported that Bev resulted in apoptosis induction of glioblastoma cancer stem-like cells with p53 upregulation and extracellular signal-regulated kinase (ERK) phosphorylation levels reduction *in vitro*. ERK phosphorylation promotes cell growth and proliferation. Other reported that Bev induce an adenocarcinoma cell line apoptosis through the mechanism of endoplasmic reticulum stress. In this study, we evaluated the difference in sensitivity to currently available chemotherapy for GBM (TMZ and Bev) between the *IDH1*-mutant and *IDH1*-wildtype types, and investigated the underlying mechanism with a high-precision comprehensive analysis using RNA-seq.

IDH1 is a cytosolic enzyme that uses NADP to oxidize citrate to α-ketoglutarate (α-KG), which generates NADPH2. *IDH1*-mutant allele converts α-KG into the oncometabolite 2-hydroxyglutarate (2-HG), and $NADPH_2$ by cytosolic IDH1 is impaired. It results in the production of considerable amounts of 2-HG, and leads to the accumulation of aberrant DNA and histone (such as H3K9me3 and H3K27me2) methylation. Moreover, a high concentration of 2-HG is also known to inhibit α-KG-dependent dioxygenases, procollagen-proline 4-dioxygenase, and hypoxia-inducible factor 1α (HIF-1α). This evidence is consistent with the present study result, that is, the expression of extracellular matrix- and vascular endothelial cell-related genes was dramatically changed in *IDH1*-mutant GBM cells after adding Bev to TMZ.

【Conclusions】

In *IDH1*-mutant U87 cells, the expression of genes for the extracellular matrix and immune cell migration increased, whereas that of cell cycle progression decreased after the addition of Bev to TMZ. Interestingly, there were no such variations in the *IDH1*-wildtype U87 cells. In support of these results, TMZ + Bev showed an antitumor effect only in the *IDH1*-mutant U87 xenograft model *in vivo*. A high-precision comprehensive analysis by RNA-seq investigated the underlying mechanism of Bev efficacy to *IDH1*-mutant GBM. Further studies of gene expression profiling in *IDH1* mutation gliomas using NGS will provide more genetic information and will lead to new treatments for this refractory disease.