主論文の要旨

Aberrant Active *cis*-Regulatory Elements Associated with Downregulation of RET Finger Protein Overcome Chemoresistance in Glioblastoma

RET finger protein の発現低下に伴う異常なアクチブシス制御因子が膠芽腫の化学療法抵抗性を改善する

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【Introduction】

H3K27ac status regulates cis-regulatory elements, which encourages gene transcription. HDACs deacetylate lysine residues on core histones, leading to a decrease in gene transcription. In cis-regulatory element regions, complexes with HDAC repress histones by H3K27ac deacetylation.

Glioblastoma is the most aggressive brain tumor with a survival rate of only 14.6 months. Temozolomide(TMZ) is the mainstream drug, but with a 5-year survival rate in 10% of cases. MGMT demethylation leads to TMZ resistance. PARP1 also contributes to TMZ resistance by the BER pathway. PARP-binding protein (PARPBP) is increased in various types of cancer.

RFP or TRIM27 is a transcription factor that becomes oncogenic upon fusion with RET tyrosine kinase. RFP is involved in cellular processes like cell growth and apoptosis, and is expressed in many types of cancer. RFP forms a tripartite complex along with Histone deacetylase 1(HDAC1) and NF-Y. Downregulation of RFP leads to the disruption of the RFP-HDAC1-NF-Y complex, subsequently increasing thioredoxin binding protein-2 (TBP-2/ TXNIP), which further leads to an increase in oxidative stress in cancer cells. This phenomenon has been observed to also increase chemosensitivity of cisplatin in cancer cells.

We studied whether RFP downregulation caused TMZ chemosensitivity in TMZ-resistant gliomas, and whether the breakage of the RFP-HDAC1 complex had a negative effect on active cis-regulatory elements mediated by H3K27ac.

【Materials and methods】

Following transfection of T98, U87-MGMT and TGS-01 cells with siRFP using Lipofectamine 3000, and TMZ treatment, the number of cells was counted using a Countess™ automated cell counter.

U87-MGMT RFP-KD cells were injected in 5-week-old female BALB/c nu/nu mice. Following TMZ treatment, specimens were evaluated by Kaplan Meier survival analysis.

ChIP-seq samples were prepared for U87-MGMT and T98 cells transfected with siRFP using H3K27ac antibodies. Miseq or Hiseq sequences were trimmed with Trim Galore! v0.4.4, evaluated by FastQC and aligned to hg19 by Bowtie2 v2.2.9. HOMER was used to identify RoEs and align data.

Also, RNA extracted from RFP depleted U87-MGMT and T98 cells were used to prepare libraries for RNA-seq. The sequences obtained were trimmed with Trim Galore! v0.4.4, evaluated by FastQC and aligned to hg19 by TopHat v2.1.1. Expression values were calculated using Cufflinks v2.1.1 and compared to the control group using Cuffdiff. HOMER was used to align data.

Gene expression data was obtained from UCSC Cancer Genomics Browser. We used the latest clinical data from The Cancer Genome Atlas (TCGA) for the analysis of overall survival of patients with glioma.

CellROX reagents were used to detect oxidative stress levels in RFP-depleted U87-MGMT cells with TMZ. Mitotracker reagent was used to detect the source of ROS. Propidium Iodide staining was done for cell cycle analysis. TUNEL assays were performed for the apoptosis analysis.

【Results】

Compared to the MGMT-null U87 cell line, RFP expression was high in MGMT-expressing and TMZ-resistant glioma cell lines T98, U87-MGMT and TGS-01. We transfected siRFP into T98, U87-MGMT and TGS-01cells and confirmed the downregulation of RFP by western blotting and qPCR. Assessment of the cell viability of T98, U87-MGMT and TGS-01 transfected with siRFP, showed that the combination of siRFP and TMZ treatment yielded the lowest cell viability. The combination of siRFP and TMZ also had a consistent effect on the survival of mice with the U87-MGMT orthotropic xenograft. We then performed a rescue assay showing that the addition of a plasmid (pFlag-RFP) on Day3 recovered cell proliferation in glioma cells treated with siRFP on Day1. Thus, we inferred that siRFP increases the efficacy of TMZ in RFP-expressing glioma cells (Figure 1).

We transfected U87-MGMT and T98 cells with the pFlag-RFP expression plasmid and analyzed the immunoprecipitates made with the anti-FLAG antibody western blotting, which showed that RFP forms a complex with HDAC1 in both cell lines. We located regions of enrichment (RoEs) with the marker H3K27ac of active *cis*-regulatory elements via ChIP in U87-MGMT and T98 cells that had undergone RFP-KD. We determined the corresponding gene expression levels by RNA-seq. GO analysis demonstrated that RFP-KD upregulates apoptosis and downregulates the pathways for cell division, the cell cycle and DNA replication in glioma cells. The gene expression levels and H3K27ac peaks of thioredoxin binding protein-2 (TBP-2, TXNIP) and FOXO1 in U87-MGMT cells were both activated upon RFP-KD, with an increase of more than 1.5-fold while that of PARPBP was significantly decreased. Upon ChIP-qPCR, we found significant coenrichment of HDAC1 and RFP in the “H3K27ac gain” regions adjacent to the indicated genes, while there was no significant enrichment of RFP but significant enrichment of HDAC1 in the “H3K27ac loss” regions adjacent to the indicated genes.

We thereby deduced that RFP-KD impairs cis-regulatory-elements-mediated regulation of genes related to cell division, the cell cycle, DNA replication and apoptosis (Figure 2).

We analyzed the overall survival of 633 patients with glioma which was stratified by expression of RFP [$\log_2$(RPKM + 0.001), cutoff = 10.39 (median)) by means of a Kaplan-Meier plot. Also, we found that PARPBP expression correlates with poor prognosis in patients with glioma $\log_2$(RPKM + 0.001), cutoff = 5.115 (median) through analysis via a Kaplan-Meier plot.

This shows that RFP and PARPBP expression correlates with poor prognosis in patients with glioma (Figure 3).

We found that oxidative stress was induced in U87-MGMT cells treated with siRFP and/or TMZ, upon staining with an oxidative stress detection reagent. The primary source of ROS was outside the mitochondria, probably the cell membrane. Also, the percentage of cells in the S and G2/M phase decreased and the percentage of cells in the G0/G1 phase increased, in the group of cells treated with siRFP alone. TMZ alone increased the percentage of cells in the G2/M phase. RFP depletion alone caused cell cycle suppression, independent of TMZ-treatment.

Analysis for apoptotic cell death by using the TUNEL assay revealed that a combination of siRFP and TMZ induced apoptosis (Figure 4).

【Discussion】

While the disruption of the RFP-HDAC1 complex led to the increase of H3K27ac in most cis-regulatory element regions, decreases in H3K27ac was also seen in other regions, and the precise mechanism of this remains unknown. We found that the 'H3K27ac loss' regions correlated to genes with functions of cell cycle, apoptosis and DNA replication. We performed cell cycle and apoptosis assays that confirmed this.

As high RFP expression was seen in TMZ resistant cell lines expressing MGMT, we thought it possible that RFP might be in lieu with MGMT in contributing to TMZ resistance. Our findings that TMZ sensitivity was synergistic in T98 and TGS-01 cells upon RFP-KD, but additive in U87-MGMT cells led us to consider other ways of chemoresistance that through MGMT.

PARPBP/PARP1 is a key BER agent causing TMZ resistance. We observed that RFP depletion led to a significant decrease in PARPBP expression. Also, it has been observed that PARP1 inhibitors sensitize antitumor agents in other

cancers by inhibition of thioredoxin and ROS generation. RFP-KD, and subsequently the disruption of the RFP-HDAC1 complex, results in an increase in FOXO1 and TBP-2 and generates ROS and inducing apoptosis. Our findings showed increased oxidative stress in U87-MGMT and T98 cells subjected to RFP-KD with TMZ treatment.

【Conclusion】

We conclude that the downregulation of RFP or the disruption of the RFP/HDAC1 complex leads to an increase in TMZ efficacy in glioblastoma by changing histone modifications which lead to changes in cell division, cell cycle and apoptosis.