STAT1 is a modulator of the expression of frequently rearranged in advanced T-cell lymphomas 1 expression in U251 cells

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Abstract. Aberrant expression of frequently rearranged in advanced T-cell lymphomas 1 (FRAT1) contributes to poor prognosis in a number of carcinomas. However, its role in glioma remains controversial. In the present study, gene expression profiling was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO) functional enrichment and ingenuity pathway analysis (IPA) to evaluate the differential expression of genes and proteins in FRAT1 knockdown U251 glioma cells in comparison with the control. Western blot analysis was conducted to assess the expression levels of FRAT1 and STAT1. A total of 895 down-regulated genes were identified in FRAT1-silenced U251 cells. The most enriched processes determined by GO and KEGG analysis of the 895 differentially expressed genes were associated with proliferation, migration and invasion. According to IPA, significant canonical pathways, including the interferon, hepatic fibrosis and Wnt/β-catenin signaling pathways, were identified to be the major enriched pathways. The elevated expression of STAT1 in U251 cells was validated. These results highlighted the regulatory role of FRAT1 in glioma cells with upregulated STAT1 expression.

Introduction

Glioma is the most common type of primary intracranial tumors in adults and is associated with a poor prognosis (1-6). The majority of clinical studies neglected the evaluation of survival time after the change in the World Health Organization definition that was put forward in 2000 (7-11). Although a limited number of patients (2-5%) survive >3 years (reported in 2007) (12,13), the median survival time of most patients is only 15 months (reported in 2012) (14). Currently, the standard treatment for patients with glioma involves surgical resection, followed by a combination of the chemotherapy drug temozolomide and radiotherapy (15,16). Despite the effective treatment strategy, the prognosis for glioma remains poor, with a median survival period of ~14.6 months and a 3-year survival rate of 10% (reported in 2009) (15). In contrast to therapies developed for other types of cancer, simple and small improvements have been made in the treatment of glioma over the recent decades; the pathophysiology of glioma remains to be clearly elucidated, and the discovery of novel molecular targets is imperative for the advanced therapy of glioma.

The frequently rearranged in advanced T-cell lymphomas 1 (FRAT1) gene is a protooncogene that was first cloned from T-cell lymphoma (17). FRAT1 acts as a positive regulator of the Wnt/β-catenin pathway (18,19) and is able to suppress glycogen synthase kinase-3 (GSK-3)-mediated phosphorylation (18,20). High expression of FRAT1 has been identified in breast, cervical, ovarian, esophageal and non-small cell lung cancer, suggesting its crucial role in malignant tumors (21-26). In addition, FRAT1 knockdown has been demonstrated to inhibit the expression levels of β-catenin, cyclin D1 (CCND1) and c-myc in hepatocellular carcinoma cells under hypoxic conditions (27). A previous study has suggested that FRAT1 may be a useful molecular marker for diagnosis by acting as a prognostic indicator of glioma, and a promising candidate protein for glioma therapy (28). Although FRAT1 expression has been identified to be associated with glioma, further understanding of the detailed molecular mechanisms is required in order to improve the efficacy of conventional therapeutic regimens.

Research focusing on the genome level of diseases has become increasingly common due to the continuous advancements in biotechnology. Gene expression profiling provides an insight into the process of tumorigenesis and has been identified as an efficient method for the identification of pathogenic genes (29). Based on a recent study on the protumorigenic role of STAT1 in glioblastoma (30) and a previous study (28), FRAT1 was identified as a novel target biomarker in glioma. The aim of the present study was to elucidate the potential association between STAT1 and FRAT1 expression and to analyze the expression levels of STAT1 in glioma cells by gene expression profiling.

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Materials and methods

Cell culture. Tumor cells were used to construct glioma samples as previously described (28). According to the same study (28), FRAT1 was highly expressed in U251 cells. Thus, in the current study, U251 cells were selected to observe the expression of STAT1 and investigate the mechanism of FRAT1 in glioma. U251 cell lines were purchased from the American Type Culture Collection, cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and maintained in a humidified incubator (CO_2 water-jacketed incubator; Thermo Electron Corporation) at 37°C in an atmosphere of 5% CO_2 and 95% air.

Transfection. Transfection was performed according to a previously described method (28). To generate stable FRAT1 knockdown cell lines, the pRNAT-U6.1/Neo plasmid (GenScript), which carries the green fluorescence protein gene, was selected for the expression of short hairpin RNAs (shRNAs) targeting FRAT1. The DNA oligonucleotides (Biomics Co., Ltd) with the sense and the antisense shRNAs sequences separated by a 9 bp spacer and having *Bam*HI and *Hin*dIII compatible overhanging ends were fused to linearized pRNAT-U6.1/Neo plasmid. All of the plasmids were confirmed by sequencing. After constructing the shRNA plasmid, the most potent plasmid for the knockdown of FRAT1 was identified, designated as pRNAT-FRAT1, which was used to knock-down FRAT1 expression in subsequent experiments.

In gene transfection, $2x10^5$ U251 cells per well were plated onto 6-well plates and grown overnight to 60-70% confluence. Subsequently, these cells were transfected with pRNAT-FRAT1 and empty pRNAT-U6.1/Neo vector using Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) under standard conditions. According to the multiplicity of infection, the amount of transfected plasmid was 0.7 μl of $6x10^8$ TU/ml. Untransfected parental cells were used as control for stable selection. Fluorescence microscope (Olympus micropublisher 3.3RTV; Olympus Corporation) were used to observe the expression of green fluorescence protein, the cells with fluorescence ratio of ≥80% were used for subsequent experiments. And the time interval between transfection and subsequent experimentation was 168 h. The FRAT1 sequences were as follows: 5'-GAGCTGGCAAGC AGGGCAT-3', 5'-AGCTAGTGCTCTCTGGAAA-3' and 5'-GCAGTTACGTGCAAAGCTT-3'.

Gene expression profiling and hierarchical clustering analysis. Total RNA was extracted from U251 cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), followed by purification on an RNeasy column (Qiagen, Inc.). The integrity of RNA was assessed using a bioanalyzer (Agilent Technologies, Inc.). The total extracted RNA (100 ng) was labeled and hybridized to Human Gene 1.0 ST microarrays (Affymetrix; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The differential expression of genes were calculated based on the log_2-fold change between the normal U251 cells samples (4448-1, 4448-2, 4448-3) and FRAT1 knockdown (4449-1, 4449-2, 4449-3) U251 cells samples. Bidirectional hierarchical clustering was conducted for differentially expressed genes (DEGs) (31).
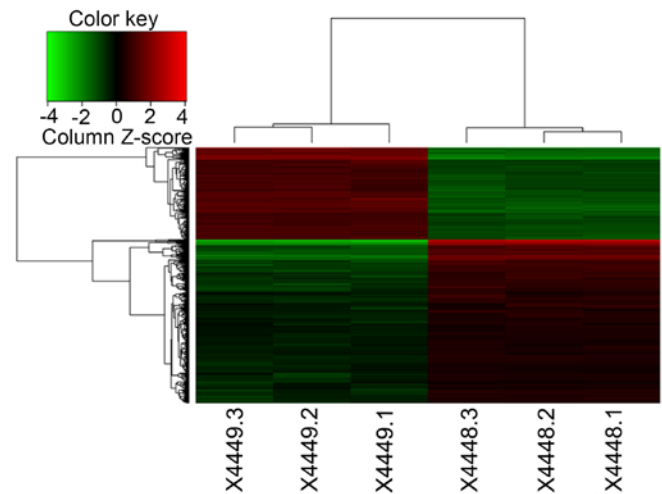

Figure 1. Hierarchical cluster analysis of FRAT1 knockdown and normal glioma cells. The horizontal axis represents sample names; 4448-1, 4448-2 are 4448-3 are normal cells; 4449-1, 4449-2 are 4449-3 are knockdown cells. The left vertical axis represents clusters, and the above horizontal axis represents clusters of samples. Red indicates upregulated genes and green indicates downregulated genes. FRAT1, frequently rearranged in advanced T-cell lymphomas 1.
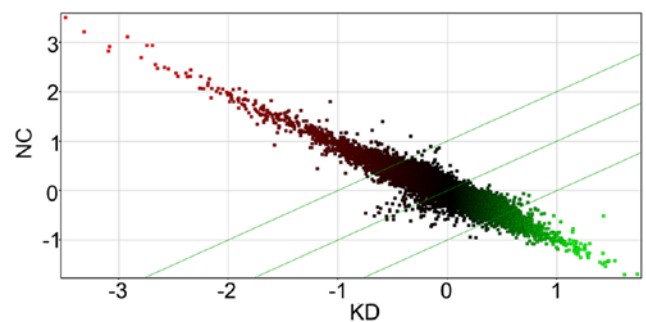

Figure 2. Scatter plot of gene expression variation between FRAT1 knockdown and control cells. The top green line represents upregulated genes in control compared with FRAT1 knockdown cells. The bottom line represents downregulated genes in FRAT1 knockdown cells compared with control. FRAT1, frequently rearranged in advanced T-cell lymphomas 1; NC, normal cells; KD, knockdown.

Enrichment analysis. Gene ontology (GO; http://www.geneontology.org/) terms were displayed as a significant network using the BiNGO plug-in of Cytoscape software (version 3.2.1) (32). The Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) database was used to identify significantly enriched pathways with a false discovery rate (FDR) <0.05 (33). The Database for Annotation Visualization and Integrated Discovery was used to identify the enriched functions with FDR<0.05 set as the significance cut-off level (34).

Ingenuity pathway analysis (IPA). The list of DEGs between FRAT1 knockdown and control FRAT1 U251 cells, which contained gene identifiers and corresponding expression values, was uploaded to the IPA software (Qiagen, Inc.) (35). IPA was used to evaluate the differentially expressed data associated with metabolic pathways, molecular networks and biological processes. Each gene identifier was matched to its
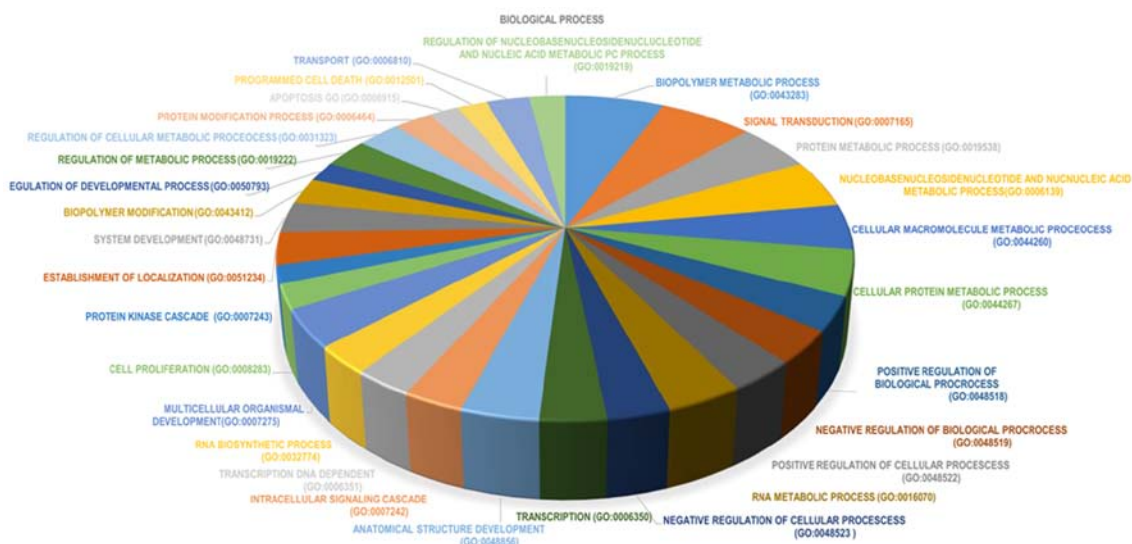
Figure 3. Significantly enriched Gene Ontology terms of differentially expressed genes between normal U251 cell and FRAT1 knockdown U251 cells based on biological process. The numbers represent the count of altered genes in a certain category together with the corresponding Gene Ontology ID number. FRAT1, frequently rearranged in advanced T-cell lymphomas 1.

corresponding gene object in the ingenuity pathway knowledge base (http://www.ingenuity.com).

Western blot analysis. Western blot analysis was performed as previously described (28). Briefly, cells were harvested and lysed, and the cleared lysates (30-50 µg/well) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gel) and transferred onto nitrocellulose membranes (EMD Millipore). After blocking for 2 h at room temperature in Tris-buffered saline (TBS; pH 7.4) with 0.1% Tween 20 (TBS-T) containing 5% non-fat dry milk, the membranes were incubated with primary mouse anti-FRAT1 (diluted 1;200; cat. no. ab108405; Abcam) and mouse anti-STAT1 (diluted 1;500; cat. no. ab3987; Abcam). Membranes were then washed in PBS-T and incubated with horseradish perox-idase-conjugated anti-mouse secondary antibody (diluted 1;5,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. The signals were detected using enhanced chemiluminescence detection solution (Pierce; Thermo Fisher Scientific, Inc.). Protein expression was calcu-lated by densitometry using the Scion Image software (Scion Corporation).

Results

DEGs between control and FRAT1 knockdown U251 cells. U251 cell lines were used to identify DEGs in glioma cells and to explore the changes in gene expression. A total of 1,388 genes were identified as DEGs between normal and FRAT1 knockdown cells. Of these DEGs, 493 were identified as upregulated and 895 as downregulated in FRAT1 knock-down cells compared with control U251 cells. Hierarchical cluster analysis revealed that the three clusters of FRAT1 knockdown cells were distributed within the FRAT1 knock-down cells and that the three glioma samples (4449-1, 4449-2 and 4449-3) were within the glioma sample cluster (Fig. 1). In addition, the generated scatter plot also demonstrated the

differential genes expression in FRAT1 knockdown cells in comparison with the control (Fig. 2).

Functional enrichment of DEGs. To investigate the biological functions of DEGs in FRAT1 knockdown U251 cells, GO enrichment and KEGG pathway enrichment analyses were conducted. In GO enrichment analysis, the majority of enriched GO terms were biological processes (39% of the terms). DEGs were mainly enriched in the following biological processes: i) 'Biopolymer metabolic process'; ii) 'signal transduction'; and iii) 'protein metabolic process' (Fig. 3). The top 10 significantly enriched terms in biological processes were also identified (Fig. 4). In the cellular compo-nent group, DEGs were primarily enriched in the following terms: i) 'Nucleus'; ii) 'cytoplasm'; and 'organelles' (Fig. 5). In order to conduct a comprehensive analysis, the top 10 significantly enriched cellular component terms were also identified (Fig. 6). In the molecular function group, DEGs were significantly enriched in: i) 'DNA binding'; ii) 'transcrip-tion factor activity'; and iii) 'receptor binding' (Fig. 7). The top 10 significantly enriched terms in molecular function are presented in Fig. 8. The results of the KEGG pathway analysis revealed that differentially expressed genes mainly enriched in the prostate cancer and cytosolic DNA-sensing pathways following FRAT1 knockdown in glioma cells (Fig. 9).

IPA. To further identify the key genes and pathways involved in FRAT1 knockdown cells and establish the associations among these genes, IPA was performed. Based on IPA, several path-ways that were associated with enriched genes were identified. 'Interferon signaling' was the most enriched pathway, and the activation of this pathway was significantly inhibited (Fig. 10). A diagram presenting the genes involved in the inter-feron (IFN) signaling pathway was generated (Fig. 11). The results demonstrated that the IFN signaling pathway may be a major pathway of FRAT1 activity in U251 cells, and demon-strated that STAT1 may act as a crucial downstream molecule
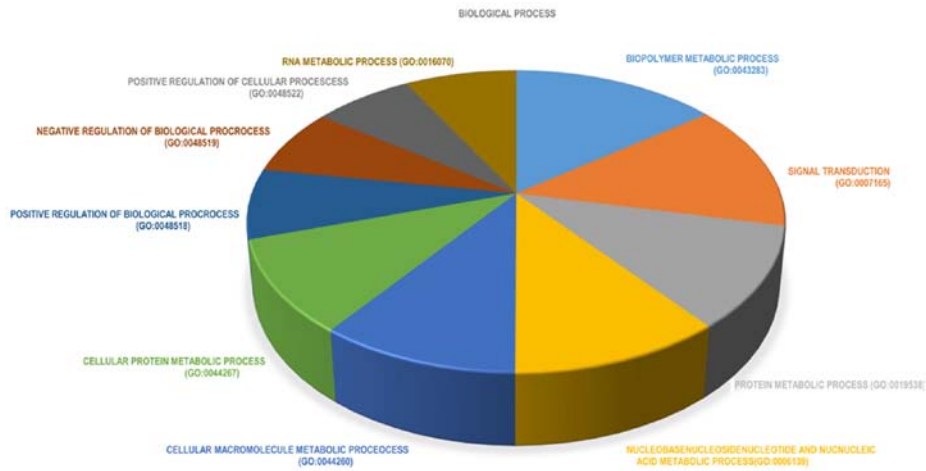
Figure 4. Top 10 significantly enriched Gene Ontology terms of differentially expressed genes between normal U251 cell and FRAT1 knockdown U251 cells based on biological process. The numbers represent the count of altered genes in a certain category together with the corresponding Gene Ontology ID number. FRAT1, frequently rearranged in advanced T-cell lymphomas 1.
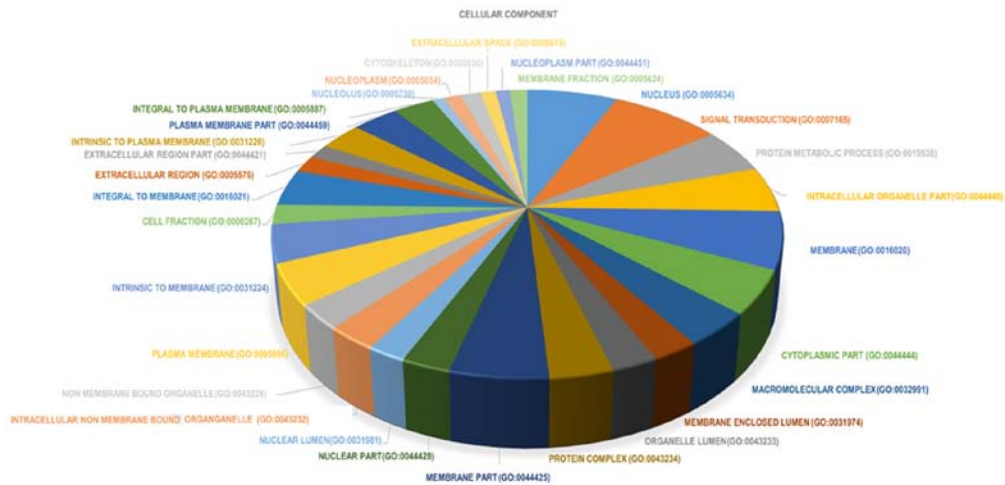

Figure 5. Significantly enriched Gene Ontology terms of differentially expressed genes between normal U251 cell and FRAT1 knockdown U251 cells based on cellular component. The numbers represent the count of altered genes in a certain category together with the corresponding Gene Ontology ID number. FRAT1, frequently rearranged in advanced T-cell lymphomas 1.
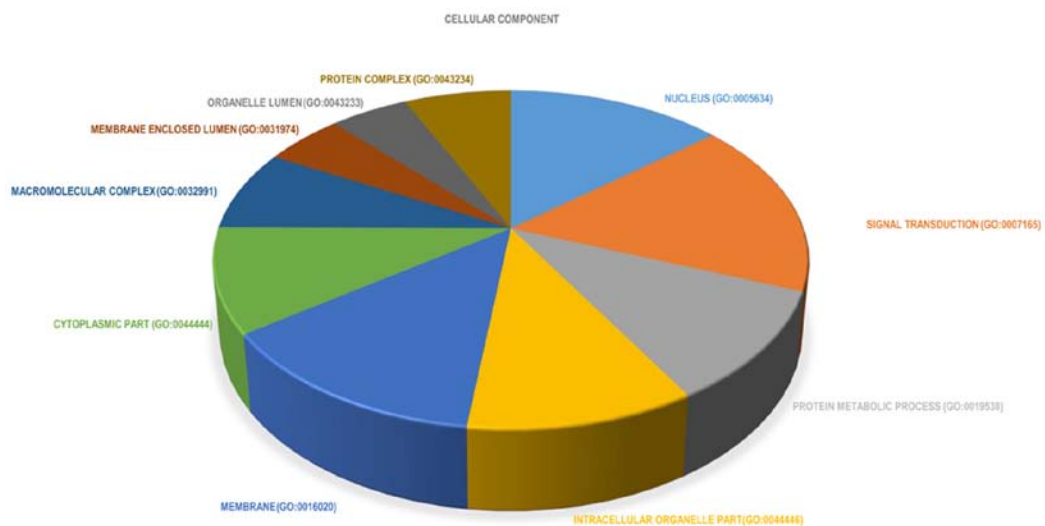

Figure 6. Top 10 significantly enriched Gene Ontology terms of differentially expressed genes between normal U251 cell and FRAT1 knockdown U251 cells based on cellular component. The numbers represent the count of altered genes in a certain category together with the corresponding Gene Ontology ID number. FRAT1, frequently rearranged in advanced T-cell lymphomas 1.
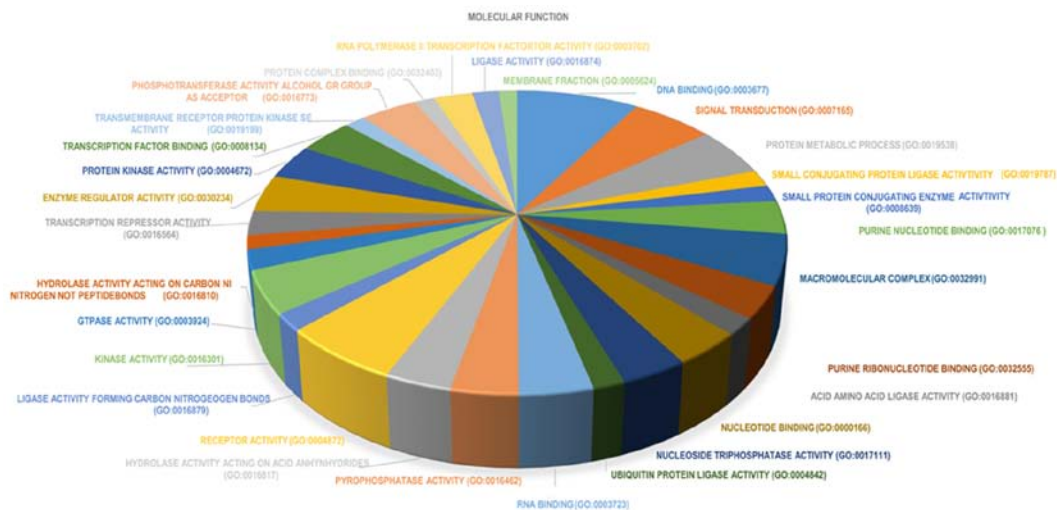
Figure 7. Significantly enriched Gene Ontology terms differentially expressed genes between normal U251 cell and FRAT1 knockdown U251 cells based on molecular function. The numbers represent the count of altered genes in a certain category together with the corresponding Gene Ontology ID number. FRAT1, frequently rearranged in advanced T-cell lymphomas 1.
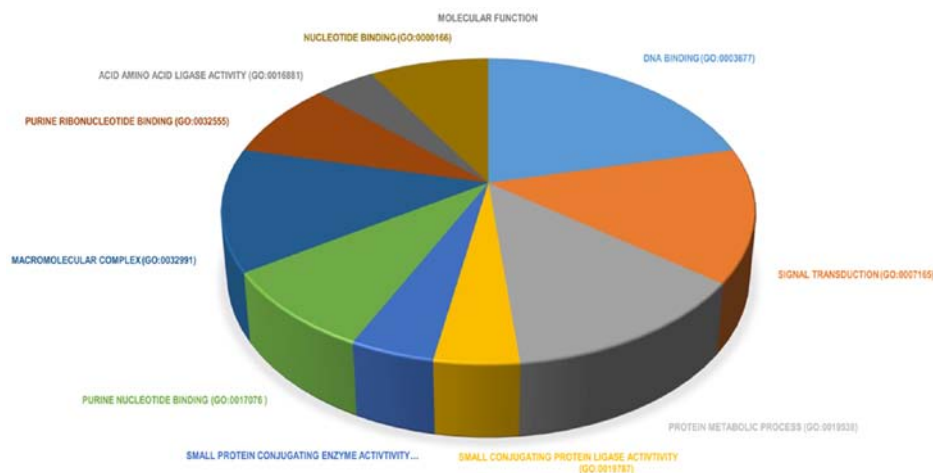

Figure 8. Top 10 significantly enriched Gene Ontology terms of differentially expressed genes between normal U251 cell and FRAT1 knockdown U251 cells based on molecular function. The numbers represent the count of altered genes in a certain category together with the corresponding Gene Ontology ID number.
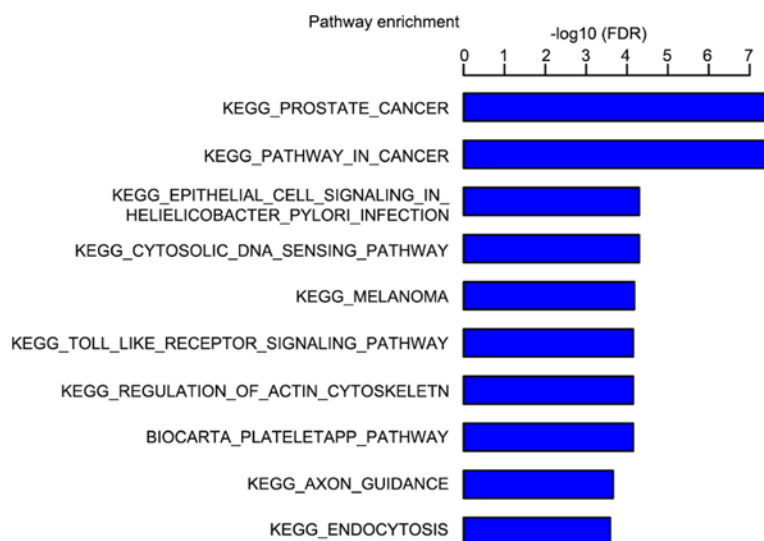

Figure 9. Functional pathway enrichment of differential genes analyzed using the KEGG database. The top 10 significantly enriched pathways based on a P<0.001 are presented. FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes.
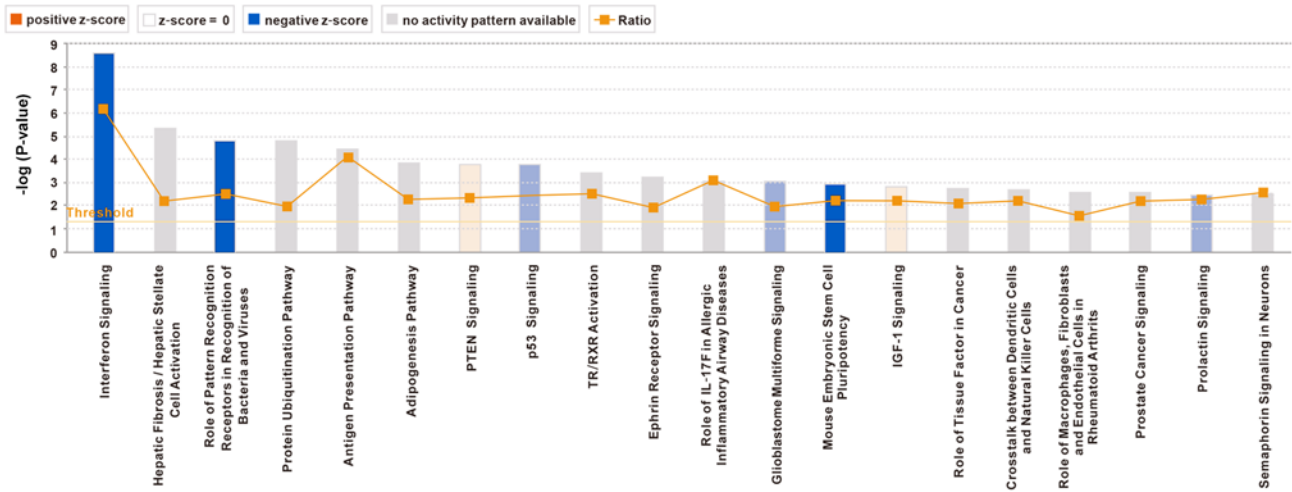
Figure 10. Ingenuity pathway analysis of significant canonical pathways associated with differentially expressed genes. 'Ratio' represents the ratio of genes from the submitted gene list to the total number of genes in the pathway. The yellow horizontal line indicates threshold for significance (P=0.05).
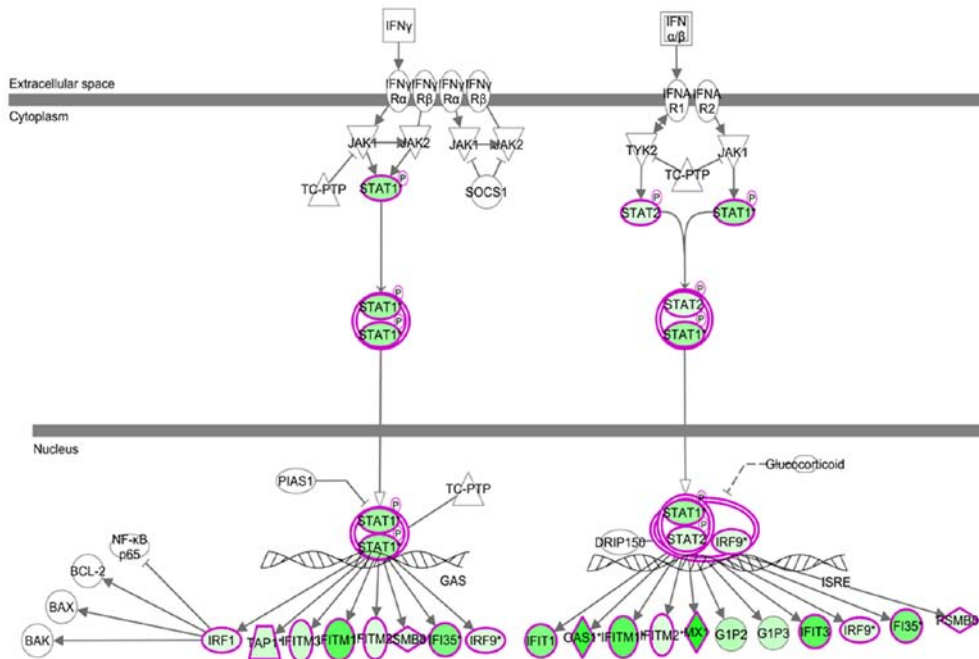

Figure 11. Ingenuity pathway analysis of functional relationships among genes involved in the IFN signaling pathway. Green represents downregulated gene expression in FRAT1 knockdown cells. IFN, interferon; FRAT1, frequently rearranged in advanced T-cell lymphomas 1.

of FRAT1. The expression level of STAT1 was downregulated in FRAT1 knockdown cells.

FRAT1 improves the expression of STAT1 in U251 cells. To further explore the role of FRAT1 in the expression of STAT1 in glioma cells, FRAT1 knockdown clones and control cells were used to analyze STAT1 protein expression by western blot analysis. As presented in Fig. 12, FRAT1 and STAT1 protein expression was significantly down-regulated in FRAT1 knockdown clones compared with the levels in untreated cells. Thus, this experiment validated the study hypothesis that FRAT1 regulated the expression of STAT1 protein in U251 cells, suggesting the involvement of FRAT1-regulated STAT1 modulation in the occurrence and progression of glioma.
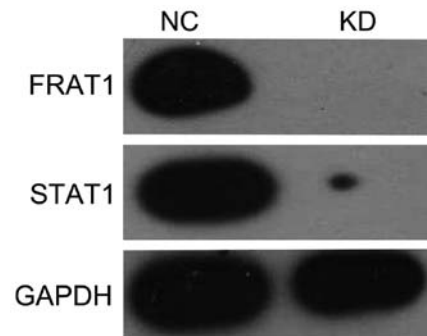

Figure 12. Protein expression of FRAT1 and STAT1 in normal U251 cells and FRAT1 knockdown U251 cells evaluated by western blotting. NC, normal cells; KD, knockdown; FRAT1, frequently rearranged in advanced T-cell lymphomas 1.

Discussion

FRAT1 is one of the main components of the Wnt signaling pathway and has been considered to positively regulate the Wnt/β-catenin signaling transduction pathway via the dissociation of GSK-3β from axin and inhibition of β-catenin phosphorylation (36-39). As a result, unphosphorylated β-catenin allows nuclear translocation and transcription activation of Wnt target genes, including c-myc and CCND1, thus leading to subsequent abnormal cancer cell proliferation (40). Recently, upregulation of FRAT1 has been identified in several types of cancer and is believed to serve a role in cancer invasion, metastasis and other malignant phenotypes (41). A number of studies have demonstrated that FRAT1 serves a major role in tumor progression (21-26). Previous studies revealed that low expression of FRAT1 resulted in the inhibition of human glioma cell proliferation, migration and invasion (28,42). In the current study, a total of 1,388 genes significantly differentially expressed between normal U251 and FRAT1 knockdown U251 cells were identified. Of these, 493 were upregulated and 895 were downregulated following FRAT1 knockdown. Next, GO, KEGG and IPA pathway analyses were conducted. In GO enrichment analysis, the majority of enriched GO terms were in the category of biological process. In KEGG pathway analysis, the most significant pathways were associated with cancer. The results of IPA pathway analysis revealed that 155 genes were associated with immune response, and the majority of these genes were involved in the IFN pathway. In addition, the interaction of these genes in the IFN pathway demonstrated that STAT1 was one of the main modules associated with the response to the knockdown of FRAT1, the module most significantly associated with immune response.

STATs, the downstream targets of IFN, are stimulated by tyrosine phosphorylation in the C-termini (43). Previous studies have indicated that STATs contribute to the upregulation of several genes associated with tumor cell proliferation, including c-myc, pim-1 proto-oncogene, serine/threonine kinase and CCND1 (44-47). In addition, recent studies have demonstrated that pim-1 serves two distinct roles in cancer; it increases the rate of cancer cell proliferation and inhibits apoptosis (48,49). STAT1 overexpression has been reported to markedly increase the proliferation of glioma cells, whereas its suppression evidently inhibits cell proliferation (50). In addition, western blot analysis confirmed that STAT1 protein was highly expressed in U251 cells, which was reduced following the knockdown of FRAT1 in U251 cells.

The activated STAT proteins are present in various types of malignancies including leukemia, prostate cancer and neck tumors (51,52). Recent studies have indicated that STAT signaling was activated in cancer, such as in lymphoma, lung cancer and head and neck cancer, and dysregulation of this factor may contribute to oncogenesis (44,53,54). Moreover, a recent study has demonstrated that STAT1 serves a protumorigenic role in glioma (30). Furthermore, it was hypothesized that poor prognosis may be attributable to chemoresistance and/or radiation resistance of tumors that express STAT1 (30). Similarly, STATs have been identified to increase the expression of certain anti-apoptotic regulatory proteins, including the Bcl family proteins (55). STATs are considered important regulators of the development and differentiation of

multicellular organisms; STAT proteins have been suggested to have primarily evolved to mediate cytokine signaling, particularly in cells of the immune system (56). Indeed, gene knockout experiments in mice indicated a pivotal role of STAT proteins in the development and regulation of the immune system (57). Thus, STAT1 may serve important roles in glioma via immune response, including: i) Proliferation; ii) inhibition of apoptosis; iii) chemoresistance; and/or iv) radiation resistance. Therefore, targeting STAT1 may be a novel therapeutic approach for the treatment of glioma. A recent study reported that oncolytic virotherapy using herpes simplex virus type I promoted glioma regression by inhibiting STAT1/3 activity; STAT1/3-induced therapeutic resistance was inhibited and, as a result, oncolytic action was promoted (58). These findings are consistent with the results of the current study.

To the best of our knowledge, the present study suggested for the first time that FRAT1 may positively regulate the Wnt/β-catenin pathway, which in turn activates target genes, including c-myc and CCND1. STAT1 mediated the upregulation of c-myc, pim-1, CCND1 and Bcl family genes, thus enhancing the proliferation of glioma cells. In addition, low expression levels of STAT1 were identified in FRAT1 knockdown U251 cells, indicating that STAT1 expression was positively regulated by FRAT1. Therefore, it was concluded that FRAT1 acted as a positive regulator of STAT1, which led to increased glioma cell proliferation, and that the protumorigenic effect of STAT1 was mediated by FRAT1. Based on a previous study on the effects of FRAT1, more experiments analyzing the role of STAT1 in glioma should be conducted in the future to validate the results of the present study. In addition, the Wnt/β-catenin and IFN/STAT1 pathways were identified to be associated in glioma through FRAT1. Investigation of the effect of IFN on FRAT1 in glioma would be of great benefit in future studies.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Author's contributions

GG, SW and YW performed data analyses and wrote the manuscript. YH, YR, JZ and DL contributed significantly

to data analyses and manuscript revision. GG, SW and YW conceived and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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