RIT1: A Novel Biomarker in Glioblastoma

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Abstract

Background: Glioblastoma is the most common type of malignant brain tumor and the most feared cancer among adults. Based on in vitro and in vivo models, it was recently postulated that the deregulated expression in glioblastomas of key genes known to be involved in early neurogenesis could be the instigator of brain tumorigenesis.

Objectives: Our objective is to show that the expression of such genes like *RIT1* (Ras Like Without CAAX 1) which encodes an unusual "orphan" GTPase protein could be useful in early diagnosis of glioblastoma.

Methods: We surveyed *RIT1* expression and potential role in glioblastoma in-silico using various publicly available datasets.

Results: We showed a significant and progressive upregulation of *RIT1* expression in various glioblastoma datasets. *RIT1* expression ranked among the top upregulated genes (4-7%) and correlated with poor survival outcomes (HR =5.7, 95% CI). Genetic and epigenetic analysis didn't reveal significant aberrations that could underlie this deregulated expression, but its transcriptional activity could be significantly controlled by *STAT3*, one of the main players in the onset of glioblastoma.

Conclusions: This is the first report that identifies *RIT1* as an oncogene in glioblastoma paving thus the way for its potential use as a biomarker and therapeutic target.

Keywords: RIT1, glioblastoma, bioinformatics, STAT3

Introduction

Brain cancer is a significant source of cancer-related morbidity and mortality worldwide. Although the incidence of neurological cancer is rare accounting for less than 2% of the total number of cancer cases, it is considered among the most feared, aggressive and difficult-to-treat malignancies[1]. Due to the heterogeneity of these tumors they are categorized into 29 histologic groups according to the World Health Organization's (WHO) Classification of Tumors of the central nervous system (CNS)[2]. Glioblastoma Multiforme (GBM) falls under the category of malignant astrocytoma (grade IV) and is considered the most aggressive and prevalent type of gliomas, grouped as such from grade I to IV[3]. It is still almost invariably fatal even after aggressive treatment regimens that incorporate neurosurgery, radio- and chemotherapy[4, 5]. Several properties make GBM incurable despite tremendous advancement in basic research and clinical practice. Among these factors are:1- the infiltrative nature of glioma cells making the complete surgical resection impossible even with excessive advancement in neurosurgical techniques, 2-the insufficient delivery of treatments caused by pharmacokinetics, 3- the blood–brain barrier (BBB), and 4- the developing resistance to conventional chemotherapy and radiation which spares these cells from eradication[2] . It is thus imperative to unravel the detailed mechanisms of brain tumorigenesis in order to develop effective and targeted methods for intervention.

RIT1 belongs to the Ras subfamily of GTPases which are known to be implicated in regulating a variety of cellular processes, including cell growth, transformation, differentiation, morphogenesis and apoptosis[6]. Despite harboring the GTPase activity like other proteins from the same family, RIT1 lacks however the prenylation motif (CAAX, XXCC or CXC) required for its association with the plasma membrane[7]. In humans, the protein is encoded by the *RIT1* gene located on 1q22 and shares approximately 50% sequence homology with the well-studied *RAS* oncogenes. *RIT1* has a widespread occurrence in the human nervous system and its variants with gain of function mutations are highly associated with Noonan syndrome (NS), an autosomal dominant disorder (OMIM #616564). NS patients

develop several partially penetrant developmental anomalies, such as postnatal growth retardation and failure to thrive, congenital heart defects, hypertrophic cardiomyopathy (HCM), hyperkeratosis, and hypotrichosis[8]. Recently, somatic mutations in *RIT1* were shown to be present in several types of cancer including lung adenocarcinoma, hepatoblastoma, urinary tract carcinoma, and adult myeloid malignancies[7]. In mice, *Rit1* is expressed in neurogenic niches of the CNS, the sub granular zone in the dentate gyrus of the hippocampus, and the subventricular zone of the lateral ventricles. Inactivation of *Rit1* doesn't alter hippocampal development but hippocampal neural cultures derived from *Rit1*$^{-/-}$ mice display increased cell death and blunt MAPK cascade activation in response to oxidative stress[9]. On the contrary, CNS conditional mice model with active RIT1 expression stimulated adult hippocampal neurogenesis and caused an extended neural precursor cells niche[10].

Although the role of *RIT1* in brain development in mammals is well-studied, its expression pattern in human gliomas is still largely unknown. Here, we sought to survey *RIT1* expression in various cohorts and within different grades of human gliomas. We report for the first time a significantly upregulated pattern of this gene in gliomas with a potential role in the progression of the disease. *RIT1* high expression was positively associated with decreased survival rate mainly among lower grade glioblastoma patients suggesting its potential prognostic value. Lastly, we demonstrate that *RIT1* expression is positively correlated with that of Signal transducer and activator of transcription 3 (*STAT3)*, the critical mediator of tumorigenesis in GBM, and could be one of its direct targets.

Material and Methods

***mRNA expression analysis, Kaplan–Meier survival plots and correlation studies for RIT1 in TCGA data*.**

Gene expression profiling interactive tool (GEPIA; http://gepia.cancer-pku.cn/index.html) is an interactive web server that uses a standard processing pipeline for analyzing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects[11].

Boxplots were performed to visualize the expression of RIT1 in various tumors versus their matched normal tissues. For analysis we used ANOVA statistical method for differential gene expression analysis, log2(TPM + 1) transformed expression data for plotting, TCGA tumors compared to TCGA normal and GTEx normal for matched normal data, |log2FC| cutoff of 1, and a q-value cutoff of 0.01. Differential gene studies and survival analysis based on RIT1 or STAT3 mRNA level in lower grade glioma(LGG; Tumor: n=518; Normal: n=207) and glioblastoma multiforme (GBM Tumor: n=163; Normal: n=207) data in TCGA was carried on. The overall survival with a median group cutoff was displayed on the plot along with the hazard ratio (HR) and log rank P value. The correlation between the expression of *RIT1* and *STAT3* in LGG and GBM was determined using Spearman's correlation analysis.

RIT1 expression analysis in various glioma datasets

We used the Oncomine webportal to analyze *RIT1* mRNA expression in various human glioma cohorts. Oncomine (https://www.oncomine.org/resource/login.html) is an online database consisting of previously published and open-access microarray data englobing 715 datasets and 86,733 samples[12]. The mRNA expression of *RIT1* in clinical brain cancer tissue was compared with that in normal control, using a Students' t-test. The search filters were set up as RIT1 for gene name, cancer vs normal analysis, and Brain/CNS cancer for cancer type. Thresholds were set as follows: gene rank, 10%; fold change, 2; and P-value, 0.01. Analysis were done on TCGA, GSE2223, GSE4290, GSE7696 and unavailable GEOdataset by Shai et.al [13]. Using Genevestigator v3 suite we evaluated *RIT1* expression among 632 cancer categories from data selection. Level of expression of *RIT1* based on the signal intensity on Affymetrix Human Genome U133 Plus 2.0 Array was displayed. Then we extracted samples with the highest 5% expression of *RIT1* amongst all categories.

Genetic and epigenetic alterations of RIT1 in glioblastoma

The cBioPortal for Cancer genomics is an open-access resource (http://www.cbioportal.org/), providing visualization and analyzing tool for more than 5,000 tumor samples from 105 cancer studies in TCGA pipeline. The term "RIT1" was searched within the cBioPortal database and a cross-cancer summary was obtained for it. The search parameters included alterations (amplification, deep deletion, missense mutations), copy-number variance (CNV) from GISTIC and RNA seq data with the default setting. In order to see the effect of epigenetic modification i.e. DNA methylation, we analyzed the methylation pattern of *RIT1* in promoter region (mainly CpG islands) of normal vs glioblastoma specimens using the Wanderer web tool (http://maplab.imppc.org/wanderer/doc.html) [14].

Analysis of the RIT1 gene for promoter regulation by STAT3

We used the **INSECT**2.0 (**IN**-silico **SE**arch for **C**o-occurring **T**ranscription factors, version 2.0) web server for prediction and analysis of the human *RIT1* promoter for potential binding sites for the transcription factor STAT3 (https://bioinformatics.ibioba-mpsp-conicet.gov.ar/INSECT2/index.php). The chosen binding sites for STAT3 were based on the Jaspar (Murine) and Transfac (Human) databases, and the analysis include a phylogenetic assessment of the corresponding murine *RIT1* promoter.

Results

Differential expression studies of RIT1 transcript in pan-cancer

We surveyed the expression pattern of *RIT1* amongst different cancer types by *in silico* analysis of publicly available expression datasets within the gene expression profiling interactive tool (GEPIA) project constituting of RNA sequencing data from 9,736 tumors and 8,587 normal samples. Our analysis demonstrated that *RIT1* expression was significantly upregulated only in four cancer subtypes amongst which are Glioblastoma Multiforme (GBM) and Lower Grade Glioma (LGG) (Figure-1A). Studying *RIT1*

expression in 632 different cancer categories presented in Genevestigator database revealed that those with the highest expression correspond mainly to brain glioma samples (Supplementary Figure-1). Among the 5% that scored the highest RIT1 expression; 25% showed to be corresponding to gliomas. Furthermore, mRNA expression from GEPIA online database showed that *RIT1* is upregulated by 2 and 2.5-log2 fold change in LGG and GBM, respectively as compared to matched normal tissues retrieved from TCGA and Gtex (Figure-1B).

Upregulated expression of RIT1 in human gliomas relative to normal brain tissues

To further explore the differential expression of *RIT1* between glioblastoma and non-cancerous brain tissues, the TCGA data as well as three other glioblastoma cohorts were analyzed using the Oncomine online database web portal (Figure-2A). All four datasets showed a significant increase in the expression of *RIT1* in glioblastoma tissues compared to that of normal brain ($p<0.01$; Figure-2B). *RIT1* scored among the top 4% differentially upregulated genes in the TCGA-GBM dataset. This high score was also identified in additional datasets where *RIT1* scored among the highest differentially upregulated genes in GBM tissues (7% in GSE2223 and GSE4290, 2% in GSE7696)[15-17]. These results point to commonly and significant consistent overexpression of the *RIT1* in human glioblastoma.

A progressive upregulation of RIT1 and a potential prognostic value

Global transcriptional profiles of gliomas of different types and grades are distinct from each other and from that of normal brain. We thus studied the expression of *RIT1* from Shai.et.al dataset in varying pathologic types and grades of gliomas (low-grade astrocytomas (grade II), the anaplastic astrocytomas and oligodendrogliomas (grade III) and the malignant glioblastoma (grade IV)) as well as seven normal brain samples taken from the subcortical white matter[13]. The observed up-regulated pattern of *RIT1* was progressive as we move from lowest grade to more malignant form of glioblastoma (Figure-3A). These findings suggest that the up-regulation of the *RIT1* is possibly implicated in the development of

premalignant precursors and in the progression of these lesions to malignant glioblastoma. Additionally, to evaluate the possible prognostic value for *RIT1* in different grades of glioblastoma we performed Kaplan-Meier survival analysis according to the median mRNA expression of *RIT1* in both GBM and LGG using Gepia analysis tool. The results showed that high *RIT1* mRNA expression (HR =5.7, 95% CI) was associated with worse overall survival for glioblastoma patients but the results didn't reach statistical significance (log-rank P=0) (Figure-3B). When *RIT1* evaluation was separately done on GBM and LGG patients, only the latter showed a statistically significant association between high *RIT1* expression and poor overall survival (Figure-4A,B). A high *RIT1* expression score might thus be associated with decreased survival time mainly among lower grade glioma patients.

Unrevealed mechanism of action underlying the upregulation of RIT1 in GBM

Then we sought to understand the molecular mechanism responsible for upregulating *RIT1* expression in various glioblastoma stages. For this reason, we surveyed the alteration frequency of mutations and copy number alterations (CNAs) of this gene in all types of cancer using cBioportal database. Our analysis revealed only a minor contribution (0.6% of the cases) of such alterations in glioblastoma (Figure-5A). We then opted to investigate the methylation status of the promoters of *RIT1* gene using TCGA Wanderer, an interactive web viewer for the visualization of DNA methylation based on TCGA data. The results showed no significant change in the methylation pattern of *RIT1* promoter between glioblastoma and normal brain tissues underscoring the need to understand the mechanism(s) underlying the observed upregulated pattern of *RIT1* in glioblastoma (Figure-5B).

STAT3 a potential transcriptional regulator for RIT1 in glioblastoma

We used the INSECT2.0 software to assess the potential transcriptional regulation of RIT1 by STAT3 a well-established oncogenic driver of GBM. Based on TRANSFAC and JASPAR databases, we searched for potential binding sites over the upstream 2000bp promoter and 1000bp downstream regions (relative to

the transcription start site) on the *RIT1* human gene and its ortholog in mice. We set the position weight matrix (PWM) at >90%, and the results did show a total of 11 potential binding sites, two of them with an overlapping pattern in mice (Figure-6A,B). To support our finding, we showed a significant positive correlation between RIT1 and STAT3 mRNAs, both in glioblastoma(R=0.7, p=1.9e-100) and normal (R=0.81, p=5.6e-72) tissues (Cerebellum, Cortex and Hippocampus) where RIT1 is known to be highly expressed (Figure-7A,B)[18]. *STAT3* expression level was further analyzed in LGG and GBM versus normal matched TCGA and GTEx data. Consistent with previous studies, we showed a significant upregulation of *STAT3* in both grades of gliomas with a higher expression in GBM (Supplementary Figure-2). Further investigations and in-vitro studies are warranted to confirm a direct transcriptional regulation of *RIT1* by *STAT3*.

Discussion

The present study aimed at investigating the expression and prognostic value of *RIT1* in glioblastoma using integrated large databases. Previous *in-silico* and experimental studies on *RIT1* have documented its role in numerous cancer types and in the neurogenesis process, yet its expression and prognostic value in GBM was not assessed. We found that *RIT1* mRNA expression level in GBM tissues was significantly higher than that in non-tumor tissues ($p < 0.001$). Moreover, the upregulation of *RIT1* followed a progressive pattern as we move from lower grade glioma to the malignant multiforme. In contrast, *RIT1* did not tend to be altered in the GBM genomic signature, and its methylation pattern was not significantly disrupted. The higher expression of *RIT1* was significantly correlated with poor prognostic survival outcome, mainly in LGG. We finally proposed a novel molecular insight in the genetic network controlling glioblastoma by linking *RIT1* overexpression to two well-documented oncogenic players, *STAT3* and *SOX2*.

Using transcriptome sequencing, we demonstrated for the first time a significant upregulated expression of *RIT1* in glioma that scored among the top upregulated genes in various GBM cohorts. The mechanism responsible for deregulating RIT1 expression in GBM is still poorly understood. Previous studies have shown that gain of chromosome 1q, particularly in the region of 1q21-25, is the most frequent finding (29%) among childhood high grade gliomas. This is even accentuated with the finding of a significant association between 1q gain and decreased survival among these patients. Thus the authors proposed the need for the identification of one or more candidate genes specifically on that region to be the key for identifying pathways involved in the biology of these tumors and as reliable prognostic biomarker(s)[19]. It is noteworthy to mention that *RIT1* maps to 1q22 suggesting that the upregulated expression of this gene in both LGG and GBM might simply be a surrogate of the copy number gain of chromosome 1q. Forthcoming work is warranted to validate the expression of the *RIT1* subfamily in LLG and GBM using orthogonal measures (e.g., immunohistochemistry). Other potential mechanisms responsible for *RIT1* overexpression could be hypomethylation, promoter/enhancer activation by highly expressed TFs, or downregulation of miRNAs that would normally inhibit *RIT1* expression.

The frequency of genetic alterations (mutations, CNVs, deletions…etc.) in *RIT1* within GBM was not high enough ($\simeq 1\%$) to explain its consistent upregulation among all studied cohorts. Methylation was ruled out, but further studies should be carried on due to the lack of representable number of normal brain tissues in the methylation experiments. miRNAs expression profile would only be beneficial if there are experimental proofs on the direct regulation of *RIT1*. Based on the miRTarBase which provides information about experimentally validated miRNA-target interactions (MTIs), three human miRNAs were predicted to directly target *RIT1* (miR-125a-5p, miR-218-5p and miR-340-5p[20]. The three were shown to be significantly downregulated in GBM, and their role in tumor initiation, progression and invasion was well-defined[21-23]. Yet, these studies pointed out to distinct targeted genes regulated by each miRNA while neglecting their common target; *RIT1*. Further studies will be needed to

evaluate the impact of the downregulation of those miRNAs specifically on *RIT1* expression and consolidate the hypothesis about their common target in GBM tumorigenesis.

Lastly, transcriptional regulation by one or multiple TFs could be the key for understanding *RIT1* overexpression. Promoter analysis revealed a couple of binding sites for transcriptional factors that could be implicated in this regulation. Amongst these is *STAT3*, a member of the STAT family of transcription factors known to activate cytokine- or growth factor-responsive genes upon phosphorylation[24]. The consecutive activation of *STAT3* resulting from gain-of-function mutations or overexpression of upstream growth factor receptors or signaling kinases is detected in 90% of human GBM tumors and GBM cell lines[25]. Moreover, a recent study on epigenome-based malignant transformation of initially lower-grade gliomas identified *STAT3* (geometric test; p = 1e-2; fold enrichment of 4.36) to be among the top hypomethylated gene in the acquired recurrent glioma-CpG island methylator phenotype (G-CIMP) which is characterized by unfavorable overall survival relative to the G-CIMP-high methylated subtype. The activation of STAT3 drive several pro-oncogenic mechanisms that promote cell proliferation and survival, immune suppression, invasion, and angiogenesis in GBM[26]. Thus, hypomethylated and activated phosphorylated STAT3 could directly regulate the expression of *RIT1* in glioblastoma which can in turn enhance the progression of the disease by affecting its downstream modulators. Ultimately, in-vitro studies are warranted to confirm *RIT1* as a novel direct downstream target for STAT3.

In parallel, we could also hypothesize that RIT1 modulate directly or indirectly STAT3 expression and/or transcriptional activity. Indeed, the serine/threonine kinase (AKT), which is considered among the primary targets for *RIT1*-kinase function, is also proven to be a key player in stimulating the transcriptional activity of both STAT3 and SOX2 [10]. It is noteworthy to mention that AKT signaling pathway has been identified as one of the three central cell signaling pathways of GBM by The Cancer Genome Atlas (TCGA). Furthermore, a direct correlation between AKT activation and the

histopathological grade of glioma exist were 84% of GBM are positive to phosphorylated AKT (pAKT), whereas it is barely detected in healthy tissues[27]. SOX2 is a member of the Sex-determining region Y (SRY)-box (SOX) factor, a family of transcriptional regulators that are implicated in embryonic development and are considered as key components for the maintenance of stem cells in adult tissues. SOX2 plays a critical role in tissue homeostasis and regeneration through maintaining stem cell activity in several compartments, particularly in the central nervous system (CNS), in adults[28]. This transcriptional factor showed consistent overexpression in malignant glioma from several different and independent cohorts while displaying minimal expression in normal tissues[29] . SOX2 revealed to be a stemness-related gene in glioblastoma and recognized as the most enriched gene among the stemness signature in GBM stem cells (GSCs)[30]. High levels of SOX2 have been associated with tumor aggressiveness and worse prognosis. In-vitro and in-vivo studies confirmed that RIT1 activation enhance hippocampal neural progenitor cells proliferation through activating SOX2 transcriptional activity in an Akt-dependent manner[10]. Thus, *RIT1* aberrant expression and its positive correlation with *SOX2* further pinpoint to the critical role played by *RIT1* in maintaining the self-renewing properties of GBM stem cells which could be in a part through direct regulation of SOX2 expression. Taken into consideration that SOX2 was recently acknowledged to regulate in turn p-AKT and p-STAT3 in GBM , we could come up with a scenario where the 3 players RIT1, STAT3, and SOX2 are within the same positive feedback loop that is involved in GBM tumorigenesis (Figure-8). Therefore, *RIT1* silencing or inhibition might be a novel therapeutic approach and a key to combat glioblastoma through targeting several critical pathways implicated in self-renewing capacity of GCS which is responsible for tumor heterogeneity, maintenance and metastasis.

Conclusion

This study presented for the first time *RIT1* gene which encodes a RAS protein, as an oncogene in glioblastoma and opened the door for further investigation on its potential use as therapeutic target and

molecular biomarker. The RAS subfamily of GTPases created an evolution in the field of cancer. Members of this family are among the most studied oncogenes and are used in the molecular classification of various tumors. Adding *RIT1* to this list, doesn't only expand our knowledge in the field, but would have a major impact on the health system, if targeted by specific inhibitors to successfully cure glioblastoma and/or other genetic diseases where it's overexpressed like Noonan syndrome.

Ethics approval and consent to participate

All Data used in this study are publicly available.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available in the TCGA, Shai et.al, Sun et.al, Bredel et.al, and Murat et.al repositories. Links:

https://portal.gdc.cancer.gov/projects/TCGA-GBM

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2223 [15]

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4290 [16]

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7696 [17]

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AK, GN did the conception and design of the study. AK analyzed and interpreted the results. AK drafted the manuscript. GN supervised the study.

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Abbreviations

AKT: Serine/threonine kinase 1

BBB: Blood–brain barrier

CNS: Central nervous system

CNV: Copy-number variance

GBM: Glioblastoma Multiforme

G-CIMP: Glioma-CpG island methylator phenotype

GEPIA: Gene expression profiling interactive tool

GSC: GBM stem cells

GTEx: Genotype-Tissue Expression

HCM: Hypertrophic cardiomyopathy

LGG: Lower grade glioblastoma

NS: Noonan syndrome

PWM: Position weight matrix

RIT1: Ras Like Without CAAX 1

SOX: Sex-determining region Y (SRY)-box

STAT3: Signal transducer and activator of transcription 3

TCGA: The Cancer Genome Atlas

WHO: World Health Organization's

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Figures Legends

Figure 1: Expression profile for *RIT1* across cancer types

A) Expression levels for *RIT1* across 33 TCGA tumors compared to TCGA normal and Genotype-Tissue Expression (GTEx) data using the GEPIA webserver. For each TCGA tumor type (red), its matched normal and GTEx data (green) are given; T: tumor; N: normal; n: number. Y axis: transcript per million (log2(TPM + 1)). X axis: number of tumor and normal samples. ACC: adrenocortical carcinoma; BLCA: bladder urothelial carcinoma; BRCA: breast invasive carcinoma; COAD: colon adenocarcinoma; CHOL: cholangio carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma ; DLBC: lymphoid neoplasm diffuse large B-cell lymphoma; ESCA: esophageal carcinoma; GBM: glioblastoma multiforme; HNSC: head and neck squamous cell carcinoma; KICH: kidney chromophobe; KIRC: kidney renal clear cell carcinoma; KIRP: kidney renal papillary cell carcinoma; LAML: acute myeloid leukemia; LGG: brain lower grade glioma; LIHC: liver hepatocellular carcinoma; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; MESO: mesothelioma; OV: ovarian serous cystadenocarcinoma; PCPG: pheochromocytoma and paraganglioma; PAAD: pancreatic adenocarcinoma; PRAD: prostate adenocarcinoma; READ: rectum adenocarcinoma;SARS: sarcoma; SKCM: skin cutaneous melanoma; STAD: stomach adenocarcinoma; TGCT: testicular germ cell tumors; THCA: thyroid carcinoma; THYM:

thymoma; UCEC: uterine corpus endometrial carcinoma; UCS: uterine carcinosarcoma**;** UVM: uveal melanoma**.**

B) Expression level for *RIT1* in LGG and GBM in TCGA versus matching normal TCGA tissues and Gtex data presented as log2(TPM+1) scale (*p < 0.01). Tumor group: red column; non-tumor group: gray column.

Figure-2: *RIT1* analysis in different glioblastoma cohorts

Boxplots comparing RIT1 expression in normal (left plot) and cancer tissue (right plot) that were derived from Oncomine database (A). The fold change of RIT1 in various glioblastoma datasets presented (B) expressed as boxplots for the TCGA, Bredel et.al, Sun et.al, and Murat et.al datasets. X-axis of the plot represents normal vs cancer group, Y-axis represents mRNA expression in log2 median/mean centered intensity. The line in the middle represents the median value. Differences were examined statistically by two-tailed student's t-test.

Figure-3: Association of *RIT1* expression levels with three glioma grades and overall survival time

A) RIT1 expression stratified by glioblastoma grade versus normal tissues in the Shai glioblastoma dataset derived from Oncomine software.

B) Kaplan–Meier plots of survival were generated by Gepia (http://gepia.cancer-pku.cn) using the data from TCGA comparing overall survival of patients according to *RIT1* expression in LGG and GBM together. Red line indicates the samples with gene highly expressed, and blue line shows the samples with gene lowly expressed. HR, hazard ratio.

Figure-4: Low *RIT1* expression is better corelated with survival in LGG

Kaplan–Meier plots of survival were generated by Gepia (http://gepia.cancer-pku.cn) using the data from TCGA comparing overall survival of patients according to *RIT1* expression in C) LGG or D) GBM

versus matched normal tissues from Gtex. Red line indicates the samples with gene highly expressed, ad blue line shows the samples with gene lowly expressed. HR, hazard ratio.

Figure-5: Genetic aberrations of *RIT1* in cancer subtypes and its DNA methylation status in GBM

A)The alteration frequency of a *RIT*1 gene signature was determined using the cBioPortal (http://www.cbioportal.org). The alteration frequency included deep deletions (blue), amplification (red), multiple alterations (grey) or mutation (green). B) Graphics obtained from Wanderer data base (http://maplab.imppc.org/wanderer/) of all CpGs island of *RIT1* and its percentage of DNA methylation in GBM (medium of 129 samples, Red) and normal brain tissues (medium of 2 samples, blue). In x-axis the cg# indicates the position of the CpG island. * : indicates differences statistically significant between normal and tumor samples

Figure-6: **In silico analysis of the *RIT1* promoter for potential *STAT3* binding sites**

The STAT3 potential binding sites with their corresponding position (Start, End) on the human (ENSG00000143622) and murine (ENSMUS00000028057) Rit1 gene (A), are visualized over the intronic/exonic junctions (B). Negative numbers indicate positions in the pomoter region whereas positive numbers indicate regions downstream the transcriptional starting site. PWM: position weight matrix.

Figure 7: Spearman correlation analysis of *RIT1* and *STAT3* gene and mRNA expression levels.

The expression of RIT1 was positively correlated with STAT3 mRNA expression in A) normal brain tissues ($r=0.81$, $P<0.001$) and B) glioblastoma tissues ($r=0.7$, $P<0.001$).

Figure-8: Schematic model of the *STAT3-RIT1-SOX2* pathway in glioblastoma.

p-STAT3 is a well-documented upregulated active protein in GBM that could enhance *RIT1* transcription which in turn upregulates the expression of *STAT3* and *SOX2* in an AKT dependent manner. SOX2 could

exert a positive feedback promoting phosphorylation of AKT and STAT3. The activation of this loop and the upregulation of *STAT3* and *SOX2* will enhance GBM stemness and the tumorigenesis process.

Supplementary Figure-1: *RIT1* expression among 632 cancer categories analyzed using GENEVESTIGATOR.

Supplementary Figure-2 : Expression level of *STAT3* in LGG and GBM versus matching normal TCGA tissues and Gtex data presented as log2(TPM+1) scale (*$p < 0.01$). Tumor group: red column; non-tumor group: gray column. TPM, transcripts per kilobase million.

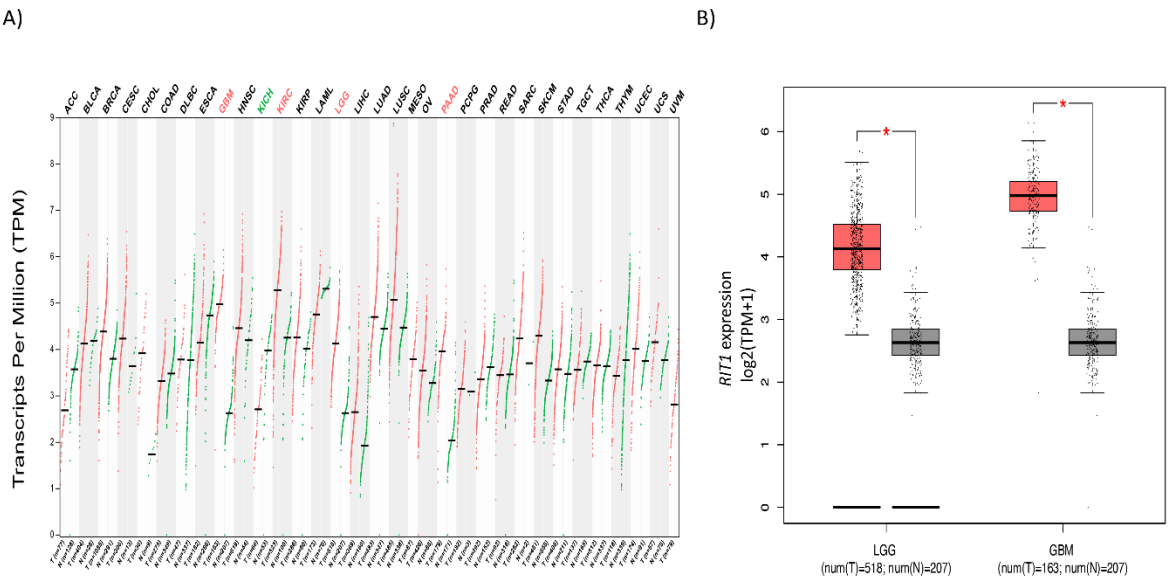
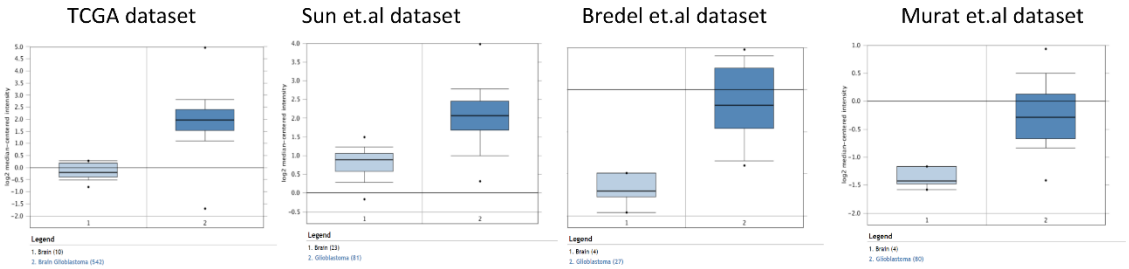

A)
B)
Transcripts Per Million (TPM)
ACC BLCA BRCA CESC CHOL COAD DLBC ESCA GBM HNSC KICH KIRC KIRP LAML LGG LIHC LUAD LUSC MESO OV PAAD PCPG PRAD READ SARC SKCM STAD TGCT THCA THYM UCEC UCS UVM
RIT1 expression
log2(TPM+1)
LGG
(num(T)=518; num(N)=207)
GBM
(num(T)=163; num(N)=207)

Figure 1

A)



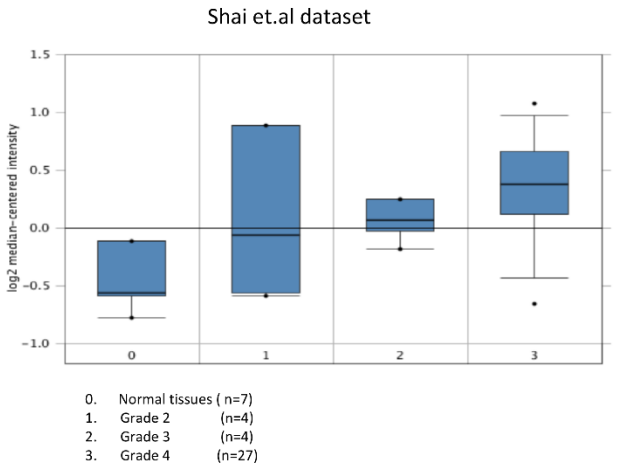
B)

Dataset Name	Glioblastoma sample size	Normal tissue sample size	t-Test	p-value	Fold change	Over-expression Gene Rank (%)	Reference
TCGA	542	10	19.153	4.96E-10	4.433	4	[15]
Bredel et.al. (GSE2223)	27	4	8.002	2.55E-5	2.047	7	[16]
Sun et.al. (GSE4290)	81	23	10.632	1.64E-16	2.293	2	[17]
Murat et.al. (GSE7696)	80	4	10.570	1.49E-5	2.206	7	[18]

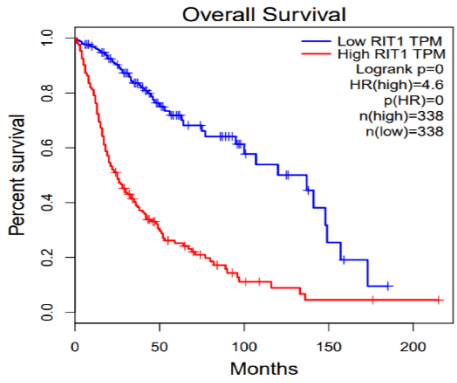
Figure 2

1

A)



B)



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9 Figure 3

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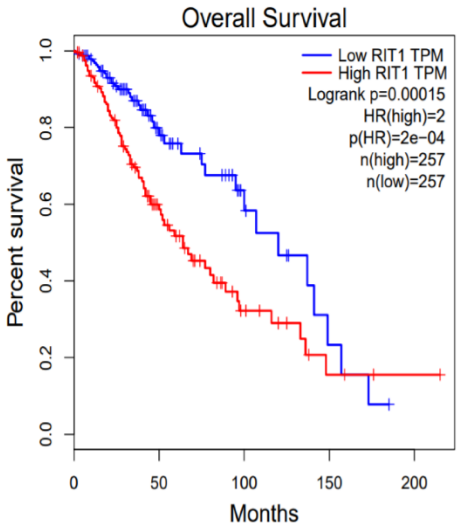
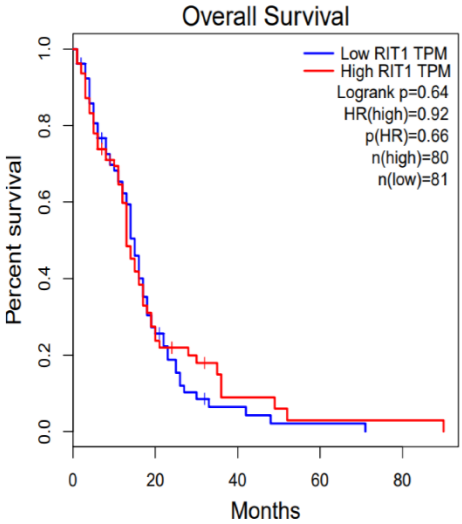
C) LGG



D) GBM



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11 Figure 4

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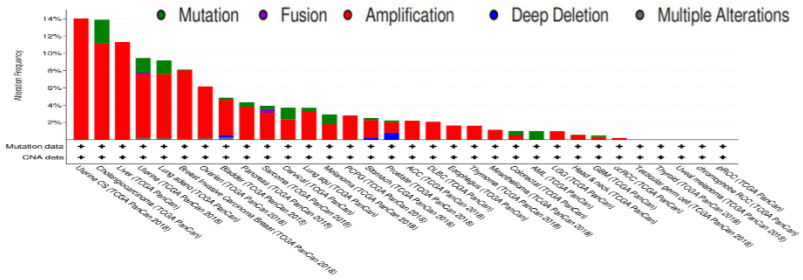
A)


Mutation
Fusion
Amplification
Deep Deletion
Multiple Alterations
Alteration Frequency
14%
12%
10%
8%
6%
4%
2%
Mutation data
CNA data

B)

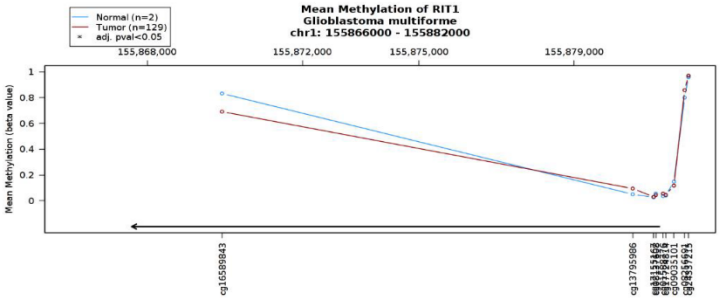
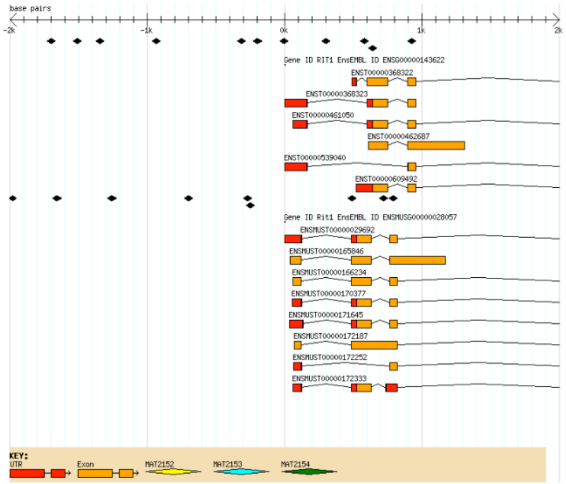

Mean Methylation of RIT1
Glioblastoma multiforme
chr1: 155866000 - 155882000
Normal (n=2)
Tumor (n=129)
adj. pval<0.05
155,868,000
155,872,000
155,875,000
155,879,000
Mean Methylation (beta value)
1
0.8
0.6
0.4
0.2
0
cg16589843
cg13795986

Figure 5

A)



B)

Gene ID	Sequence	Start	End	PMW relative Score
ENSG00000143622	GGTTTCCT	-1702	-1695	0.91
ENSG00000143622	TAGTTCCC	-1510	-1503	0.96
ENSG00000143622	TTCTTGGAAA	-1344	-1335	0.90
ENSG00000143622	TGATTCTC	935	928	0.90
ENSG00000143622	CCCTTCCT	317	310	0.91
ENSG00000143622	CCCTTCCC	-198	-191	0.96
ENSG00000143622	TCCTTCCG	-6	1	0.94
ENSG00000143622	TTCTTCCC	299	306	0.94
ENSG00000143622	CATTTCCT	580	587	0.90
ENSG00000143622	GGATTCTG	646	653	0.91
ENSG00000143622	CGATTCCC	925	932	1.00
ENSMUSG00000028057	CAATTCCC	-1988	-1981	0.99
ENSMUSG00000028057	CTGTTCCC	-1658	-1651	0.94
ENSMUSG00000028057	GTATTCCT	-1258	-1251	0.91
ENSMUSG00000028057	CTCTTCCG	-699	-692	0.94
ENSMUSG00000028057	CTCTTCCA	-272	-265	0.90
ENSMUSG00000028057	AAATTCCA	250	243	0.90
ENSMUSG00000028057	CCTTTCCC	492	499	0.92
ENSMUSG00000028057	TACTTCCT	719	726	0.93
ENSMUSG00000028057	CGATTCCC	790	797	1.00

Figure 6

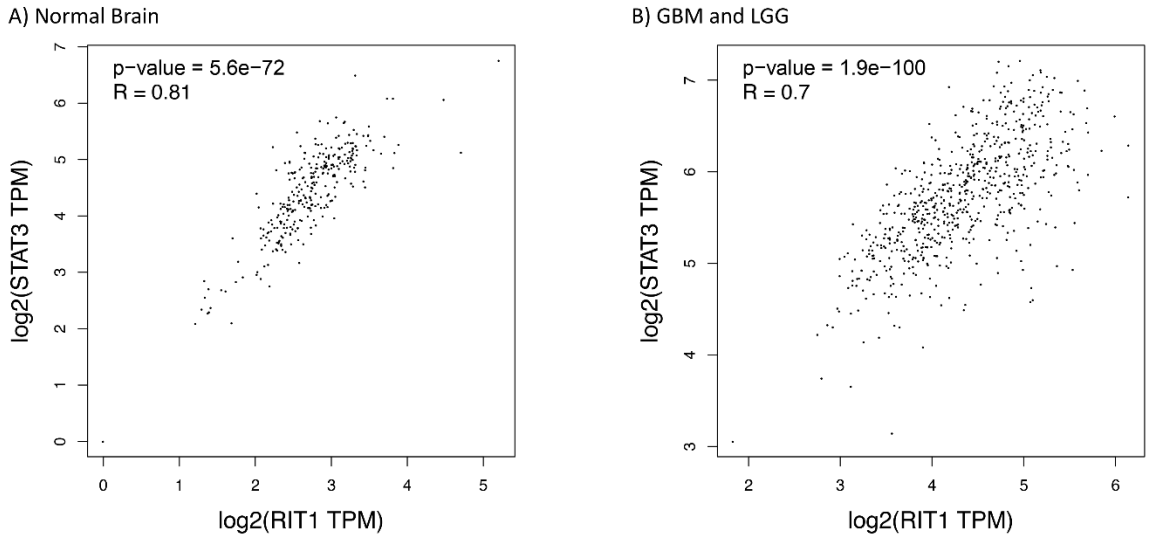

A) Normal Brain
p−value = 5.6e−72
R = 0.81
log2(STAT3 TPM)
log2(RIT1 TPM)
B) GBM and LGG
p−value = 1.9e−100
R = 0.7
log2(STAT3 TPM)
log2(RIT1 TPM)

Figure 7

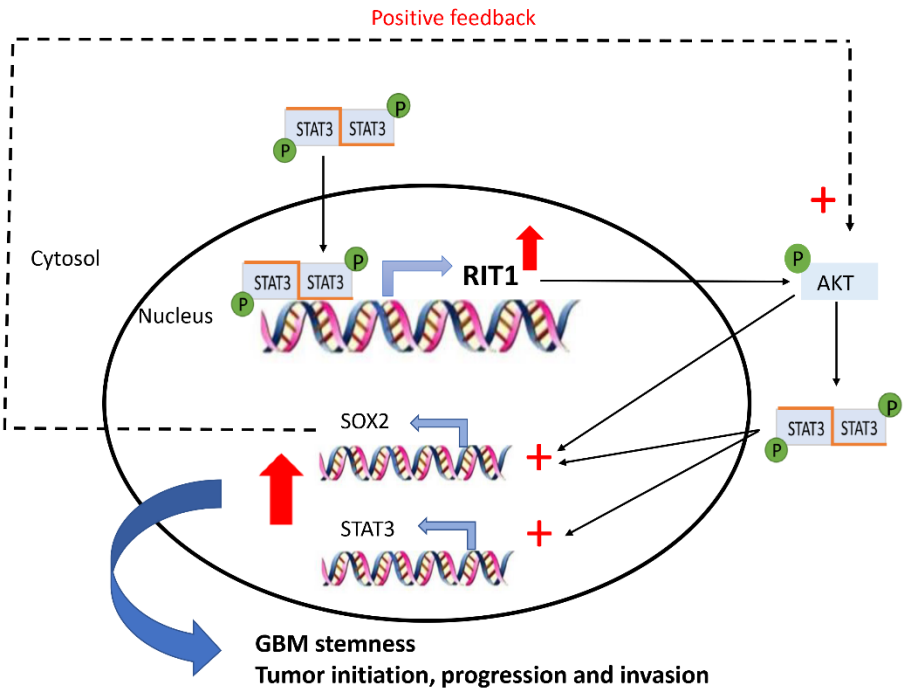
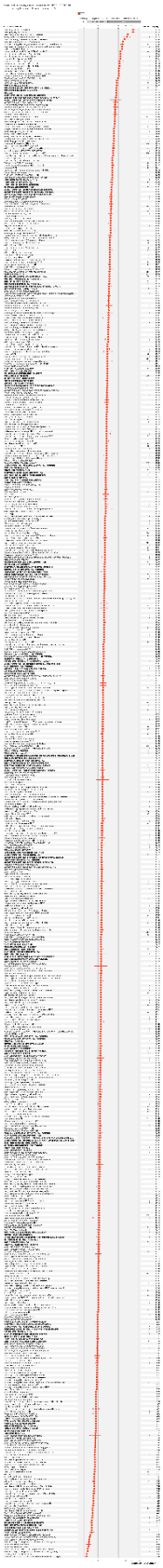
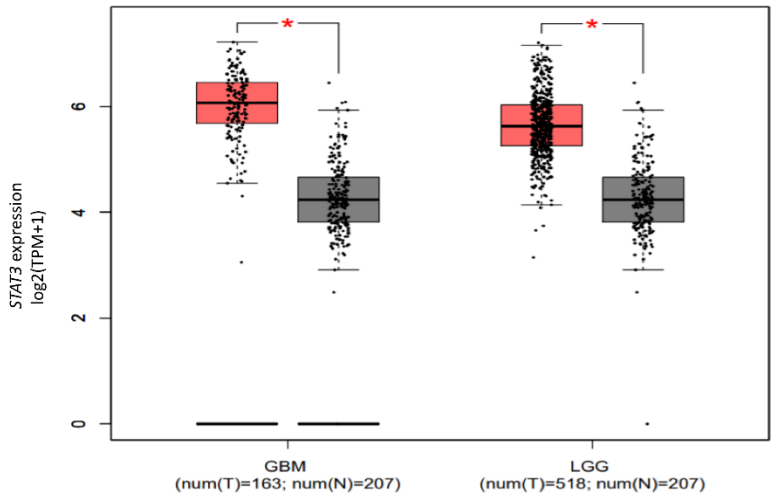

Positive feedback
STAT3
STAT3
P
Cytosol
Nucleus
RIT1
AKT
SOX2
STAT3
GBM stemness
Tumor initiation, progression and invasion

Figure 8



Supp-Figure1



Supp-Figure 2