STAT5b is a marker of poor prognosis, rather than a therapeutic target in glioblastomas

NADÈGE DUBOIS1,2, SHARON BERENDSEN1, KATHERINE TAN1, LAURENT SCHOYSMANS3, WIM SPLIET1, TATJANA SEUTE1, VINCENT BOURS2 and PIERRE A. ROBE1,2

1Department of Neurology and Neurosurgery, and The T&P Bohnenn Laboratory for Neuro-Oncology, University Medical Center of Utrecht, 3584CX Utrecht, The Netherlands; 2Human Genetics Laboratory, GIGA-Cancer Center, University of Liège; 3Department of Radiology, University Medical Center of Liège, 4000 Liege, Belgium; 4Department of Pathology, University Medical Center of Utrecht, 3584CX Utrecht, The Netherlands

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Abstract. The copy number and mRNA expression of STAT5b were assessed in samples from the TCGA repository of glioblastomas (GBM). The activation of this transcription factor was analyzed on tissue microarrays comprising 392 WHO 2016 GBM samples from our clinical practice. These data were correlated with patient survival using multivariable Cox analysis and, for a subset of 167 tumors, with signs of tumor invasiveness on the MRI. The effects of STAT5b knockdown by siRNA were assessed on the growth, therapeutic resistance, invasion and migration of GBM cell lines U87, U87-EGFRVIII and LN18 and primary cultures GM2 and GM3. The activation, but not the copy number or the mRNA expression of nuclear transcription factor STAT5b expression correlated inversely with patient survival independently of IDH1R132H status, age, Karnofsky Performance Score, treatment and tumor volume. STAT5b inhibition neither altered the cell proliferation nor reduced the clonogenic proliferative potency of GBM cells, and did not sensitize them to the cytotoxic effect of ionizing radiation and temozolomide in vitro. STAT5b inhibition significantly increased GBM cell migration, but decreased the invasion of some GBM cells in vitro. There was no correlation between the activation of STAT5b in clinical tumors and the extent of invasion on MRI OF patients. In conclusion, STAT5b is frequently activated in GBM and correlates inversely with patient survival. It does not contribute to the growth and resistance of these tumors, and is thus rather a potential prognostic marker than a therapeutic target in these tumors.

Introduction

Glioblastomas (GBM), the most common primary brain tumors, carry a dismal prognosis despite aggressive surgery, chemotherapy and radiation therapy (1,2). Subsets of GBMs, defined by the activation of given signaling pathways (for instance, c-Met) or distinct driver genetic changes (for instance, IDH1/2 mutation), however present diverse outcomes (3,4). Novel therapeutic targets and solid markers of prognosis are thus of crucial importance in the fight against these types of cancer.

STAT5 transcription factors are composed of homologous protein dimers of either STAT5a or STAT5b, encoded by two different genes, and that show both overlapping and distinct regulation, transcriptional targets and biological effects (5). By modulating the expression of effectors such as Bcl-XL, Aurora A, FAK or VEGF, STAT5 was reported to contribute to GBM growth, invasion and therapeutic resistance (6-9). The transcriptional targets of STAT5a and b present certain overlap but are also differentiated (10). In malignant gliomas, STAT5a was revealed to be activated downstream of EGFRvIII (11) and to promote cell migration, survival and proliferation, notably via induction of the long non-coding RNA LINC01198 (12,13). Similarly, STAT5b is highly expressed and predominantly activated in these tumors (6,7), notably as a result of mir-134 repression (14) and high tyrosine kinase activity (11,15). A single nucleotide polymorphism of STAT5b was reported to associate with the risk of GBM (16), and STAT5b can associate with EGFRvIII in the nucleus in GBM that harbor this mutation, which activates the transcription of Bcl-XL. STAT5b was also reported to contribute to GBM cell proliferation (7). Univariable analysis in small series of glioma patients suggested an inverse correlation between STAT5b activation and patient survival (6,8,17). It was also found that epileptogenicity in GBM associates with an improved survival and with
a decreased HIF-STAT5b activation (18). As a result, STAT5b has been proposed as a potential target for the treatment of patients with GBM (16). The nature and the specificity of the tumorigenic role of STAT5b in GBM however varies between these reports, and its clinical value as a marker of survival or a therapeutic target still needs to be confirmed in large series of patients.

Therefore, the prognostic value of STAT5b expression in a large institutional cohort of 392 GBM samples and its association with tumor invasion on the MRIs of 167 of these tumors were analyzed. The effect of STAT5b inhibition on the proliferative, therapeutically resistant and migratory capacities of cultures of human GBM was further evaluated.

**Materials and methods**

**Genetic analysis.** The GISTIC 2.0 copy number data and Agilent-based mRNA expression data of 538 and 552 GBM samples respectively of the The Cancer Genome Atlas (TCGA) repository were obtained from the UCSC Cancer Genomics Browser (accessed in September 2015). Threshold copy number (CN) values were used to perform the correlations with mRNA expression data using Pearson's correlation analyses.

**Ethics statement.** The present study was conducted following review by the ethics committee of University Medical Center of Utrecht (Utrecht, The Netherlands) and the institutional review board (IRB; TC-Bio; approval no. 16-229). According to Dutch regulations, the need for informed consent was waived for this retrospective analysis of patient clinical data.

**Tissue microarrays (TMAs), immunohistochemistry (IHC) and MR assessment.** Formalin-fixed, paraffin-embedded tumor tissues of a series of 392 GBM (320 IDH 1 R132 wild-type), and EGFRVIII (both negative) mRNA sequencing and by CGH analysis, and maintained at low passages. U87_viii cells were kindly provided by Dr M. Broekman (University Medical Center of Utrecht) and their expression of EGFRviii was confirmed by next generation sequencing. Cells were cultured in 5% CO2 in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% of 5 mg/ml penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) solution at 37°C, and maintained at low passages.

For Western blot analysis, 70% confluent cultured cells were transfected with 25 nM of Control pool non-targeting #1 (D-001810-10-05) or SMARTpool human STAT5b siRNA (M-010539-02) from Dharmacon using the DharmaFECT transfection reagent (Thermo Fisher Scientific, Inc.; DharmaFECT Transfection Reagents) according to the manufacturer's protocol at 37°C. Transfection time was 48 h, after which the cells were used for subsequent experimentations.

**Cell cultures, reagents and small interfering (si)RNA.** The genetic profile of human LNI8 GBM (cat. no. CRL-2610; ATCC) and U87 malignant glioma cells of unknown origin (cat. no. HTB-14; ATCC) was verified using CGH (Affymetrix SNP6.0 arrays) and TP53 sequencing (ion torrent). GM2 and GM3 primary GBM cells were derived from fresh samples of human GBM and cultured as previously described (20). They were characterized using GFAP IHC, TP53, IDH1/2 (both wild-type), and EGFRviii (both negative) mRNA sequencing and by CGH analysis, and maintained at low passages. U87_viii cells were kindly provided by Dr M. Broekman (University Medical Center of Utrecht) and their expression of EGFRviii was confirmed by next generation sequencing. Cells were cultured in 5% CO2 in DMEM (Thermo Fisher Scientific, Inc.) solution at 37°C, and maintained at low passages.

For Western blot analysis. Whole-cell lysate were obtained using lysis SDS 1% buffer containing protease and phosphatase inhibitors. Protein concentration was determined using BCA method (Pierce kit; Thermo Fisher Scientific, Inc.), and 25 μg of protein were loaded per lane. Western blot analysis was performed in polyacrylamide 10% gels and run for 1 h and 30 min at 100 V, then transferred to PVDF membrane (Roche Diagnostics) for 1 h at 100 V. Blocking was performed for 1 h at room temperature, in the same buffers used for the incubation of the respective antibodies. All primary antibodies were incubated overnight at 4°C and the dilution recommended by the manufacturer was used; STAT5b (Abcam; cat. no. ab194380; 1:5,000 in 4% BSA (VWR International, LLC), 1X TBS, 0.1% Tween® 20 buffer), GAPDH (Sigma-Aldrich; Merck KGaA; cat. no. 3688; 1:1000 in 4% non-fat dry milk, 1X TBS, 0.1% Tween® 20), p27 (Cell Signaling Technology, Inc.; cat. no. 3688; 1:1000 in 4% BSA 1X TBS, 0.1% Tween® 20 buffer).
Cyclin D1 (Cell Signaling Technology, Inc.; cat. no. 2922; 1:1,000 in 4% BSA 1X TBS, 0.1% Tween®-20 buffer), BCL-XL (Cell Signaling Technology, Inc.; cat. no. 2764; 1:1,000 in 4% BSA 1X TBS, 0.1% Tween®-20 buffer) and PD-L1 (Cell Signaling Technology, Inc.; cat. no. 13684; 1:1,000 in 4% BSA 1X TBS, 0.1% Tween®-20 buffer). Appropriate HRP-linked secondary antibody was used (Cell Signaling Technology, Inc.; cat. no. 7074; 1:3,000) for incubation at room temperature for 2 h with gentle shaking. For detection, enhanced chemiluminescence method was used. Clarity Western ECL Blotting Substrate (Bio-Rad Laboratories, Inc.) was used coupled with film-based imaging following the manufacturer's protocol. 

Cell survival assays. After 24 h of transfection, 2,500 cells were seeded in 96-well plate, then let to adhere overnight before to support Temozolomide (TMZ; cat. no. T2577; Sigma-Aldrich; Merck KGaA) treatment or gamma-radiation and later MTS assay (One solution cell proliferation assay; cat. no. G3582; Promega Corporation) as recommended by the manufacturer, and the absorbance was measured at 490 nm.

Clonogenic assays. After 48 h of siRNA transfection, 500 cells were seeded in six-well plate followed or not with gamma-radiation, then left to grow for 7 days and then fixed in 4% paraformaldehyde (45 min at room temperature) and stained with crystal violet (5 mg/ml) for 10 min at room temperature before counting on a light microscope (Olympus Corporation).

Migration/Invasion assays. For Boyden chamber assays, 48 h post-siRNA transfection, a calculated number of cells (50,000 for U87, GM2 and GM3; 25,000 for U87VIII and LN18) in serum-free medium with 0.1% BSA (VWR International, LLC) were seeded into the upper chamber of Transwell inserts (8 µm) coated with collagen type I (50 µg/ml) for migration and with Matrigel (500 µg/ml; precoating for 30 min at 37°C) for invasion, whereas medium with 1.5% FBS and 1% BSA was applied in the lower chamber as chemo-attractant. After 6 h of migration or 24 h of invasion, cells were fixed with 4% paraformaldehyde and stained with 0.4% crystal violet (ambient temperature, 10 min), scanned on a Hamamatsu NDP scanner, and counted. For wound healing (scratch) assays, 48 h post-siRNA transfection cells were cultured until confluence and treated for 1 h with mitomycin C (Sigma-Aldrich; Merck KGaA) and then wounded using a 100-µl pipette tip. Images of the migration distance were captured on a phase contrast Leica microscope and measured at zero time and after 1, 4, 6, 20 and 24 h and expressed in percentages of the original gap. For quantification, the margins were plotted and 3 measurements were made per scratch, and averaged to calculate the healing percentage. Each condition was performed in quadruplicate.

Statistical analysis. Statistical analysis was performed using the Prism 5.0 (GraphPad Software, Inc.) and the SPSS 24 (IBM Corp.) software. Kaplan-Meier survival estimates were obtained and multivariable Cox regression analysis was performed taking the age, KPS (> or <70), tumor volume (in cubic cm, measured with Ostrix® and based on the contrast-enhancing T1-weighted tumor boundaries), type of surgery (biopsy vs. debulking) and IDH R132H status into account to assess correlation with overall survival. Survival data were censored at 1,000 days in order to comply with the condition of proportional hazard for the survival analyses (19). Multiple t-tests and two-way ANOVA, with Dunnett’s multiple comparisons post hoc tests were performed as appropriate for non-survival data. Correlations were assessed using Pearson's test. The association of low or high Stat5b activation with MRI criteria of invasion was assessed by Chi-square and Kruskall Wallis tests. Results are expressed as the mean ± SD and a two-sided P<0.05 was considered to indicate a statistically significant difference.

Results

STAT5b activation and GBM patient survival. Agilent-based STAT5b mRNA expression values and gene copy number (CN) were obtained for 552 patients of the TCGA repository of GBM. There was a weak correlation between the mRNA expression of STAT5b and its copy number (Pearson's correlation 0.091, P=0.033), but this CN did not correlate with patient survival (data not shown). Similarly, the mRNA expression of STAT5b mRNA did not correlate with patient survival in a Cox survival model taking the Karnofsky performance score (KPS) and patient age into account (P=0.696). TMAs were obtained for a series of 392 patients treated at our neuro-oncological center and for 10 non-tumor brain tissue samples obtained from temporal lobe epilepsy surgery.

IHC showed a high level of nuclear expression of Phospho-STAT5b (p-STAT5b) in a majority of the samples, including in the non-tumor brain samples. In these non-tumor samples, 75% of non-neuronal cells presented a positive staining of the nucleus for STAT5b, i.e., an immunohistochemistry score of 3 (Fig. S1). In the GBM patients, there was an inverse correlation between the nuclear staining score for nuclear p-STAT5b (taken as a continuous variable) and overall survival in a multivariable Cox model taking the Age, KPS, tumor volume, type of surgery and IDH1R132 mutational status into account [hazard ratio (HR), 1.22; 95% confidence interval (CI), 1.11-1.346; P<0.001]. Also significant in this analysis were the patient age and KPS (Table SII for). This multivariable analysis remained even significant when the patients were dichotomized between two groups based on a practical threshold of 3, corresponding to that of ‘non-tumor’ p-STAT5b nuclear staining instead of a continuous variable (HR 1.664; 95% CI 1.290-2.150; P<0.001; Table I). Kaplan Meier survival estimates based on this threshold showed a median survival of 13.5 months in the low STAT5b activation group vs. only 9.3 months in the high STAT5b activation group of patients (P<0.001, Log Rank test, Fig. 1).

STAT5b and GBM cell proliferation. Transfection of human GBM cell cultures using a SMARTpool® of human STAT5b siRNA resulted in a significant and lasting STAT5b protein knockdown (80% protein reduction minimum) within 24 h (Fig. 2A). This depletion of STAT5b did not prove cytotoxic to any of the GBM cells as measured by a MTS test for 72 h (NS, ANOVA, n=3, Fig. 2B). In clonogenic assays, STAT5b knockdown even induced a slight but significant increase in
colony (minimum 20 cells) formation in U87 and GM3 cells (P<0.01; n=3, Fig. 2C), and did not alter that of U87VIII, LN18 and GM2 cells. STAT5b depletion also resulted in a reproducible decrease of the cell cycle inhibitor p27kip1 (Fig. 2D) and a reproducible increase of Cyclin D1 in all cell types (Fig. 2E). In search for additional effects, STAT5b inhibition did not affect expression the expression of Bcl-XL (Fig. S4), on GBM cells, and increased the expression of the immune checkpoint ligand PD-L1 (Fig. S5) in these cells.

STAT5b and chemo/radio-sensitivity of GBM cells. Given the inverse correlation between p-STAT5b nuclear expression and survival in our cohort of GBM patients and a previous study that STAT5b contributes to chemoresistance of GBM to cisplatin (12), it was investigated whether this transcription factor would also contribute to the resistance of these tumors to their conventional treatments, namely ionizing radiation and TMZ chemotherapy. In clonogenic assays performed with increasing doses of radiation (0-2-4 Gy), STAT5b knockdown
did not affect the radiation sensitivity of U87 and U87viii cells. STAT5b depletion even slightly but significantly protected LN18, GM3 and GM2 cells against radiation toxicity (P<0.01, two-ways ANOVA, n=4, Fig. 3A). The sensitivity of GBM cells to TMZ treatment (800 µM) was not significantly affected by STAT5b depletion (Fig. 3B).

**STAT5b and GBM dissemination.** In scratch assays, the closure kinetic of the monolayer gap was assessed for 24 h, and was similar between siSTAT5b and siControl in both U87 and U87VIII cells. The healing was however slightly faster in siSTAT5b-than in siControl-treated LN18 cells, lasting 20 vs. 24 h. Similarly, siSTAT5b-treated GM3 and GM2 cells healed significantly faster than their siControl counterparts (Figs. 4A and S2). Following STAT5b knockdown, the migration of U87, LN18 and GM2 cells through collagen-coated membranes of Boyden chambers increased significantly as well (by 197.7±65.4%, 201.6±11.2% and 219.7±21% respectively; P<0.001; n=3), while that of U87VIII and GM3 remained unaffected [Fig. 4B (a) and (b)].

When the Boyden membranes were coated with Matrigel however, the invasion of U87viii, GM3 and GM2 cells decreased by 53.2±17.1%, 52.2±11.9% and 43.5±15.6% upon STAT5b depletion (P<0.0001; n=4 for GM3 and n=5 for U87viii and GM2; Figs. 4C and S3), while that of U87 and LN18 did not change significantly.

Finally, in order to assess the resulting clinical effect of these diverging effects of STAT5b on migration and invasion of GBM, the available T1-weighted, gadolinium-enhanced MRI images of the first half of our patients (available in 167/196 patients) were analyzed for signs of tumor dissemination. The presence (or not) of tumor islets at a distance of the tumor mass, a ratio >2 between the maximal diameters of the T2-weighted extent of the tumor and of the contrast-enhancing T1-weighted tumor area, or invasion of the corpus callosum by the enhancing component of the tumor were assessed. There was neither any difference between the level of nuclear STAT5b staining between tumors presenting either of these dissemination features or not (N.S., Kruskall-Wallis non-parametric test), nor was there any association between a high STAT5b IHC score (>3) and these parameters (N.S., Chi-square test).

**Discussion**

STAT5a and STAT5b present overlapping and distinct regulatory pathways and transcriptional targets (5,8), and while STAT5a has been shown to favor the proliferation of malignant gliomas, STAT5b appears to be predominantly activated in GBM (7,14) and has been proposed as a therapeutic target against these tumors (16).

As previously described, indeed (6,7,17), GBM from our cohort revealed a high level of STAT5b activation. This activation was often higher than that of the glial cells...
of non-tumoral brain samples, and is inversely correlated with patient survival. The present Kaplan-Meier estimates demonstrated that tumors with a high level of nuclear p-STAT5b staining had a median overall survival more than 4 months shorter than those with a low STAT5b activation level. This prognostic association remains highly significant.
in multivariable analysis independent of the KPS, age, tumor volume, type of surgery or IDH1 R132H mutation. The present findings confirmed and extended those of previous survival analyses that were performed in monovariable fashion and on much smaller cohorts of patients (6,17). It was also observed that neither the copy number nor the mRNA expression of STAT5b correlated with survival in GBM patients, suggesting that it is truly the nuclear activation of STAT5b, rather than its mere expression, that correlates with the prognosis of patients with a GBM.

Despite its inverse association with patient survival, STAT5b activation did not support GBM cell proliferation. On the contrary in fact, the inhibition of STAT5b reduced the expression of p27 and increased that of Cyclin D1 in our panel of cell lines and primary cultures of GBM, and even slightly increased the clonogenic potential of U87 and

Figure 4. STAT5b and tumor invasion. (A) Wound healing assay after STAT5b inhibition, distance represented as percentage of wound closure. (B) (a) Transwell migration assay performed 48 h after siRNA transfection, migration expressed in percentage of siControl transfected cells after 6 h of migration; (b) representative pictures of the migration experiments. (C) Matrigel-coated Transwell invasion assay performed 48 h post-transfection. Invasion expressed in percentage of siControl transfected cells after 24 h of invasion. Data are presented as the mean ± SD, n=8 for each condition. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. si-, small interfering.
and most importantly, the effects of STAT5b on the proliferative actions of STAT5a (12,13), and likely underscores the specificity of these two STAT family members (10). Notably, Liang et al (7) observed that a less complete depletion of STAT5b than in our experiments decreased the proliferation of some GBM cell lines. This suggested that the effects of STAT5b could be concentration-dependent and non-linear, or could depend on a more complex balance with other transcription factors. It was also noted that the pro-clonogenic effect of STAT5b knockdown observed in U87 cells, was absent in U87VIII cells. EGFRvIII has been identified to form nuclear STAT5b-EGFRvIII complexes in GBM (8,21), which activate BclXL and favor cell survival (8,22), and could have contributed this difference. However, no alteration of BclXL expression was observed following STAT5b knockdown, and no sensitization of GBM cells to TMZ or ionizing radiations was observed following STAT5b inhibition as cisplatin (8). However, no sensitization of GBM cells against the cytotoxic effects of DNA-damaging agents such as cisplatin (8). However, no sensitization of GBM cells to TMZ or ionizing radiations was observed following STAT5b inhibition, and even a slight protection against these conventional anti-GBM cytotoxic agents was identified in some of the cultures of the present study. Finally, it was revealed that STAT5b inhibition also increased the expression of the immune checkpoint ligand PD-L1 on GBM cells, further casting doubts on the potential of STAT5b as a potential therapeutic target in GBM.

Collectively, the present findings clearly defined the potential of activated nuclear p-STAT5b as a prognostic marker of patient survival in GBM, independent on IDH R132H status and other classical predictors of longevity. Given its lack of significant proper oncogenic role however, our results do not support anti-STAT5b strategies as a means to treat GBM. Rather, nuclear p-STAT5b appears to be a surrogate marker of the activation of true oncogenic pathways, possibly HIF-1α (18) or tyrosine kinase receptors, which are frequently hyper-activated in GBM (8,11,24) and can, besides the JAK/STAT cascade, activate known oncogenic pathways such as the ERK/MAP kinases or the NF-kappaB pathways (3,14,20,25).

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Availability of data and materials

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

Authors’ contributions

Conceptualized the study, ND and PAR wrote the prepared and wrote the draft. ND and PAR revised and edited the manuscript. ND, TS, LS, KT, SB, WS and PAR performed data acquisition, experiments and analysis. PAR and VB supervised the study, PR, VB and TS acquired funding. All authors read and approved the final manuscript. PAR, VB and ND confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was conducted following review by the local ethical committee and the institutional review board (TC-Bio; approval nos. 16-229 and 16-342). According to Dutch regulations, the need for informed consent was waived for this retrospective analysis of patient clinical data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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