Pathology – Research and Practice 212 (2016) 517–523

Contents lists available at ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original article

Nuclear unphosphorylated STAT3 correlates with a worse prognosis in human glioblastoma

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ARTICLE INFO

Article history:
Received 8 October 2015
Received in revised form 4 February 2016
Accepted 8 March 2016

Keywords:
STAT3
Glioblastoma
Glioma
Immunohistochemistry
u-STAT3

ABSTRACT

Glioblastoma (GBM) is currently the most aggressive form of brain tumor identified, and STAT3 is known to play an important role in gliomagenesis. Moreover, while several studies have used pharmacological approaches to modulate STAT3 activity, the results have been contradictory. In this study, expressions of STAT3, pSTAT3 (Y705), and pSTAT3 (S727) were evaluated using immunohistochemistry assays of tissue microarrays containing non-neoplastic tissue (NN, n = 12), grade II astrocytomas (n = 33), grade III astrocytomas (n = 12), and GBM (n = 85) specimens. In GBM specimens, STAT3 was overexpressed and exhibited greater nuclear localization compared with lower grade astrocytomas and NN. Conversely, nuclear localization of pSTAT3 (Y705) and pSTAT3 (S727) exhibited a similar phenotype in both GBMs and NNs. MET was also detected as a non-canonical pathway marker for STAT3. For tumors with higher levels of STAT3 nuclear localization, and not pSTAT3 (Y705) and pSTAT3 (S727), these specimens exhibited increased levels of MET expression. Thus, a non-canonical pathway may mediate a proportion of the STAT3 that translocates to the nucleus. Moreover, tumors which exhibited greater nuclear localization of STAT3 corresponded with patients that presented with lower rates of recurrence-free survival and overall survival. In contrast, the phosphorylated forms of STAT3 did not correlate with patient survival. These findings suggest that phosphorylation-independent mechanisms may mediate the nuclear translocation and activation of STAT3. Further studies are needed to identify the mechanisms involved, especially those that provide targets to achieve efficient inhibition and control of GBM progression.

1. Introduction

Astrocytomas are the most frequent primary brain tumors diagnosed in adults, and are hypothesized to have a glial origin [1]. The World Health Organization (2007) classifies astrocytomas based on histological features and malignancy grade. Regarding the latter, grades I and II represent low grade, or benign tumors, while grades III and IV represent high-grade, or malignant tumors [2]. Grade I or pilocytic astrocytoma is a pediatric tumor which represents a different entity and behaves differently from other astrocytomas [3]. On the other hand, grade IV astrocytomas are known as glioblastoma (GBM), the most common and malignant astrocytoma subtype identified to date. The incidence of GBM has been found to increase with age, and peaks between 75 and 84 years of age. Furthermore, GBM is more common in males than females, and in ethnically white populations compared with populations of African descendent [4]. Currently, the treatment for GBM involves maximal surgical resection, followed by radiation therapy with concurrent and adjuvant Temozolomide (Stupp regimen) [5]. Despite this aggressive, multimodal treatment strategy, however, the prognosis for GBM remains poor. For example, the median patient survival rate is 14 months, and the >3-year survival rate is 10% [6,7]. Thus, accurately identifying key molecular events during

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http://dx.doi.org/10.1016/j.prp.2016.03.001
0344-0338/

Table 1
Clinical and histopathological information.

Histology (WHO, 2000)	Number of samples	Median of age (years ± SEM)	Gender (%)		Median of overall survival (months ± SEM)
			Female	Male	
Grade II	33	33.0 ± 2.7	52	48	67.0 ± 12.2
Grade III	12	35.0 ± 3.1	42	58	61.0 ± 18.6
GBM	85	56.0 ± 2.0	40	60	09.3 ± 3.4

tumor progression is of utmost importance in order to improve the prognosis for patients with malignant astrocytomas.

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family which includes seven members. STAT3 activation leads to the expression of genes that control angiogenesis, cell proliferation, cell differentiation, and survival [8]. Common to all STAT proteins, STAT3 is activated via tyrosine phosphorylation in response to a variety of cytokine receptors (such as gp130), receptor tyrosine kinases (such as EGFR), and non-receptor kinases (such as Src and ABL). In particular, phosphorylation of Y705 triggers the dimerization of STAT3 via phosphotyrosine-SH2 domain interactions [9]. STAT3 can form homodimers or heterodimers with STAT1, and these dimers then translocate to the nucleus to bind interferon γ (gamma)-activated sequence (GAS) DNA-binding sites [9,10]. STAT3 can also be phosphorylated on serine 727 by several proteins, including protein kinase C (PKC) and CDK5, which leads to maximal transcription activity for STAT3 [11,12]. Physiologically, STAT3 activation is tightly controlled and is usually rapidly deactivated [13]. However, in GBM, STAT3 has been found to be constitutively activated, thereby affecting cell proliferation, apoptosis, invasion, and immunosuppression [8]. Regarding the latter, the transcriptional activity of STAT3 modulates cytokine expression and regulation of MHC class II and co-stimulatory molecules. Thus, STAT3 remains active in immune cells such as natural killer cells, macrophage, and neutrophils and inhibits their anti-tumor activity [14]. Consequently, STAT3 activation facilitates the development of glioblastomas.

Based on the crucial role that STAT3 plays in GBM, numerous preclinical models of STAT3 inhibition have been established. These have included direct inhibition of STAT3 by targeting tyrosine and serine phosphorylation, nuclear translocation, dimerization, and transcriptional activation. Indirectly, inhibition of targets upstream of STAT3 signaling (such as tyrosine kinases inhibitors) has also been employed [15].

STAT3 inhibitors that directly affect the phosphorylation or dimerization of STAT3 have been shown to impair tumor growth and invasion in GBM cell lines. However, effective anti-tumor activity of STAT3 in animal models of human tumors remains controversial [15]. For example, there is evidence to indicate that unphosphorylated STAT3 forms a complex with unphosphorylated NFκB to regulate a distinct and diverse set of genes such as MET, a receptor tyrosine kinase for the hepatocyte growth factor receptor, which is overexpressed and amplified mostly in GBM and not in astrocytomas Grades II and III [16,17]. Furthermore, in addition to its transcriptional activity, a cytoplasmic form of unphosphorylated STAT3 was found to affect autophagic pathways based on its interactions with PKR kinase and repression of eIF2A phosphorylation [18]. Thus, changes in STAT3 that affect posttranslational modifications or its cellular localization may mediate different pathways of tumorigenesis.

In the present study, expressions of nuclear STAT3, pSTAT3 (Y705), and pSTAT3 (S727) were evaluated in astrocytomas and non-neoplastic specimens. It is hypothesized that a better understanding of the STAT3 activation process in GBM may facilitate the development of more effective drugs for the treatment of these tumors.

2. Material and methods

2.1. Sample selection and tissue microarrays (TMAs)

Samples collected and stored between May 1980 and December 2004 from astrocytomas grades II, III and glioblastoma patients used in this study were collected, processed and distributed by the A C Camargo Biobank [19]. Twelve brain samples from patients surgically treated for epileptic syndrome were provided by the Department of Pathology, Federal University of Parana, Brazil. The chosen areas had overall structure preserved with no significant neuronal loss and mild atypical reactive chances. The mesial sclerosis areas were not included.

Samples collected and stored between May 1980 and December 2004 were processed following approval from the A.C. Camargo Cancer Center Research and Ethics Committee (Process 1692/12).

According to criteria published by the World Health Organization (WHO), a total of 33 grade II astrocytomas, 12 grade III and 85 GBM samples (Table 1) were evaluated and compared to 12 non-neoplastic specimens. None of the patients were treated prior to surgery where tissue was collected. Although this cohort was previously described and revised by two pathologists [20], hematoxylin/eosin (H&E) slides from all non-neoplastic tissue as well as astrocytomas samples were reviewed by a third pathologist to confirm each diagnosis.

Tissue samples were fixed in buffered 4% formalin, embedded in paraffin, and used for tissue microarray construction as described [21]. A slide with a representative tumor was selected, and an area of tumor was circled on the slide. Using a TMA technology (Beecher Instruments, Silver Spring, MD), 1-mm punch was collected from representative tumor areas from each case, and these were organized into a TMA. This TMA was subsequently sectioned (4 μm) and mounted on glass slides.

2.2. Immunohistochemistry (IHC)

TMA slides were deparaffinized with an incubation at 60 °C for 12 h, followed by three successive immersions in xylene (5 min each) and rehydration through a series of graded alcohols. For antigen retrieval, slides were incubated in 10 mM citrate buffer (pH 6.0) at 96 °C for 30 min. To block endogenous peroxidases, slides were incubated in 3% H_2O_2 diluted in distilled water, and were then washed in distilled water. Slides were subsequently incubated with anti-STAT3 antibodies (Cell Signaling Technology, Danvers, MA, USA) (1:100), anti-pSTAT3 (Y705) antibodies (M9C6, Cell Signaling Technology) (1:100), anti-pSTAT3 (S727) antibodies (Cell Signaling Technology) (1:100), and anti-MET antibodies (8F11, Novocastra) (1:25) that were diluted in 1% bovine serum albumin in PBS. After 18 h at 4 °C in a humidified chamber, slides were incubated with secondary antibodies in a two-step procedure (Advance HRP; Dako, Glostrup, Denmark). After slides were washed twice with PBS for 5 min each, the slides were incubated with DAB solution for 5 min. DAB was subsequently removed by rinsing with distilled water, and the slides were counterstained with hematoxylin. After slides were dehydrated in ethanol, cleared with xylene, and mounted using Entelan™, bound antibody was examined. On each slide, a positive control for each antibody tested was included.