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Research article

Morphological and quantitative analysis on α-tubulin modifications in glioblastoma cells

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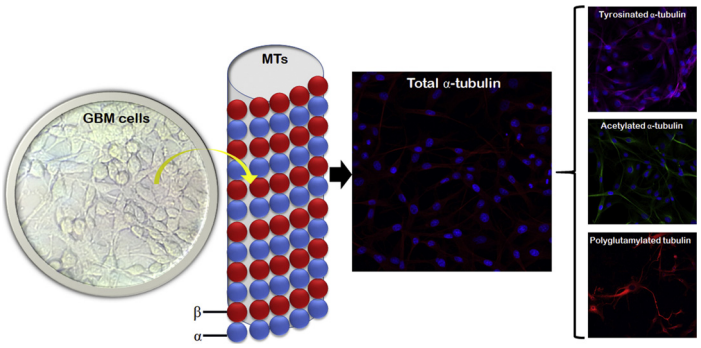
GRAPHICAL ABSTRACT



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ABSTRACT

Glioblastoma is one of the primary tumors of the brain with high invasiveness and lethality. In the study of its pathophysiology in recent years much attention has been paid to the microtubular network, but exclusively to β-III tubulin, whose presence in glioblastoma cells is associated with the degree of malignancy and diffusion. As is well known, the microtubular network performs its many functions thanks to various post-translational modifications, most of which affect the α-tubulin subunit. These modifications, able to coexist in the same microtubule, bind certain driving and cargo proteins, deeply influencing cellular functions. Since there are no data in the literature about the diverse post-translational modifications of tubulin in glioblastoma cells, this work aims to fill this gap. In the present work, through immunofluorescence, morphological analysis and Western blot, we studied the pattern of tyrosinated, detyrosinated, delta 2 (Δ2), acetylated and polyglutamylated tubulin. We detected good immunopositivity in fluorescence for almost all the modifications examined. Only Δ2 showed a very low signal. Western blot displayed that the most abundant tubulin modifications in glioblastoma cells were tyrosinated, acetylated and polyglutamylated. Morphological evaluation revealed that these modifications were more present along the cytoplasmic extensions, less evident around the cell body and always strongly evident in the mitotic spindles of the dividing cells. For the first time, the most abundant post-translational modifications and their cellular compartmentalization in glioblastoma cells have been highlighted, suggesting a novel approach in the study of their microtubular network and in the search of new experimental therapeutic strategies.

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1. Introduction

Glioblastoma, or glioblastoma multiforme (GBM), is a highly malignant brain tumor belonging to the astrocytoma family, the most frequent and most malignant form of primary brain cancer in adults [1–3]. Given its infiltrative nature and the poor prognosis that often accompanies it, numerous therapeutic efforts were made to block the growth and spread of this cancer. The main target of many therapeutic agents is represented by the microtubular network where they may block microtubule polymerization dynamics, leading to the interruption of cell division, mitotic arrest and induction of apoptosis [4,5]. In eukaryotic cells, it is well known that microtubules (MTs) are involved in essential processes such as cell division, motility, maintenance of cellular shape and intracellular transport. They are formed by a globular heterodimeric protein of 100 kDa, consisting of two monomers: α tubulin and β tubulin [6]. The heterodimers are assembled together with a "head-to-tail" arrangement, aimed at forming a polarized protofilament which interacts laterally with other protofilaments, forming a cylindrical hollow structure of about 25nm: the microtubule. The primary sequence and structure of tubulin proteins and, consequently, the properties and architecture of microtubules are highly conserved in eukaryotes. Despite this, tubulin is subject to heterogeneity, generated in two ways: i) the expression of different tubulin isotypes; ii) post-translational modifications (PTMs). Tubulin PTMs are chemical changes, mostly reversible on the C-terminal tails at the end of the heterodimer, affecting mainly the α-tubulin subunit [7]. Although fourteen different types of PTMs are known, the most characterized are tyrosination, detyrosination, delta 2 (Δ2), acetylation, polyglutamylation and polyglicylation [8–10]. Some of these modifications characterize highly dynamic microtubules (tyrosination), while others (detyrosination, acetylation and Δ2) are more present in the microtubules provided with high stability [11]. It has been established for years that the various modifications can coexist even in the same microtubule and that each is able to attract certain driving proteins and move certain effectors, creating the so-called "tubulin code" [12]. Examining the scientific literature, most of our knowledge of the post-translational modifications of tubulin comes from studies on neurons, where the microtubular network and the "tubulin code" reach maximum complexity. On the contrary, data from in-depth studies on tubulin PTMs in the glial cells are in short supply [13,14]. Despite the discovery that several post-translational modifications of tubulin, such as acetylation, tyrosination, detyrosination and polyglutamylation, have a crucial role in different cancers [15–17], this type of approach, related to the tubulin code, is still missing in the literature on glioblastoma. In fact, most work on the subject of glioblastoma focuses on aspects related mainly to tubulin β III and its possible role in the malignancy of this tumor, beside possible therapeutic approaches [18,19]. Therefore, considering the importance of the tubulin code, and given the scarcity of data on the composition and distribution of the diverse tubulin PTMs in glial cells and their potential importance in the pathophysiology of gliomas, this work aims to fill this knowledge gap on glioblastoma cells.

2. Materials and methods

All chemicals in this study were obtained from commercial. In details, all primary antibodies and the secondary AP conjugated antibodies, were purchased from Sigma Aldrich and Millipore, now Merck Millipore. (Merck KGaA, Darmstadt Germany). The Alexa Fluor secondary antibodies were purchased from Termo Fisher Scientific (Termo Fisher, Waltham, MA, US). The reagents for the MTT assay were purchased from Molecular Probes (Molecular Probes Europe BV, NE). For other chemicals, the commercial origin will be specified in the text, when necessary.

2.1. Cell culture

Undifferentiated C6 rat glioblastoma cells (American Type Culture Collection, Rockville, MD, USA) were grown in phenol-red-free RPMI-1640 medium supplemented with 10% heat-inactivated newborn calf serum, 2 mM L-glutamine, 100 units/ ml penicillin G and 100 µg/ml streptomycin sulfate in an atmosphere of 5% CO2, at 37 °C and incubated for 36 h. Depending on the methods used, the cells were cultured either in 25/75 cm^2 flasks (for Western blot) or in eight-well chamber-slides (for immunofluorescence staining procedures).

2.2. Proliferation assay

In order to analytically evaluate the growth rate of the cell line, we performed an MTT (3- (4,5-dimethylthiazol-2-yl) -2.5-diphenyltetrazolium bromide) cell proliferation assay according to the manufacturer's indications. Briefly, 5 × 103 cells were seeded in 200ul medium onto 96-well microtiter plates and evaluated at 6, 12, 24, and 36 h after seeding. At the end of each period, the medium was aspirated and replaced with 100 µl of fresh medium plus 10ul of MTT and incubated at 37 °C for up to 4 h. Subsequently 100 µl of a 0.01 M HCl / SDS solution was added to each well and incubated for up to 12 h. MTT was reduced by the proliferating cells in a colored soluble formazan. The plates were then read for absorbance, by an automatic microplate reader set to 570 nm (FluoStar Omega, BMG Labtech, Gmbh, Germany).

2.3. Indirect immunofluorescence staining

Immunopositivity for glial cell markers and tubulin PTMs was assessed by indirect immunofluorescence. The cells were cultured under the same conditions described above on eight-well chamber-slides. We use three chamber-slides for each tubulin PTMs examined. Cells were fixed in 4% PBS/paraformaldehyde (20 min) and incubated overnight with the following primary antibodies diluted in 3% PBS / BSA + 0.1% triton X 100: monoclonal anti-total α-tubulin (dil.1:1000, catalog # T6199, clone DM1A, biological source: mouse); monoclonal anti-tyrosine α-tubulin (1:1000, catalog # T9028; clone, TUB-1A2, biological source: mouse); monoclonal anti-detyrosinated α-tubulin (1: 1000; catalog # MAB5566; biological source: mouse); monoclonal anti-acetylated α-tubulin (1:1000; catalog # T7451; clone 6-11B-1; biological source: mouse); monoclonal anti-polyglutamylated tubulin (1: 1000; catalog # T9822; clone B3; biological source: mouse); polyclonal anti-tubulin Δ2 (1:1000; catalog # AB3203; biological source: rabbit); polyclonal anti-GFAP (1:1000; catalog # SAB4300647; biological source: rabbit); monoclonal anti-vimentin (1:1000; catalog # SAB4200716; clone, VIM-13.2; biological source: mouse); monoclonal anti-βIII tubulin (1:128; catalog # SAB4700544; clone, TU-20; biological source: mouse). After incubation, the cells were washed in PBS, and then incubated for 1 h at 37 °C with the following secondary antibodies: goat anti-Mouse IgG AlexaFluor 488, (1:400, catalog # A-11001); goat anti-Rabbit IgG AlexaFluor 488 (1:400, catalog # A32731); goat anti-Mouse IgG AlexaFluor 594 (1:400, catalog # A-11032); goat anti-Mouse IgG AlexaFluor 532 (1:400, catalog # A-11002); goat anti-Mouse IgG AlexaFluor 633 (1:400, catalog # A-21050). In two of the eight wells of the chamber-slides the corresponding primary antibodies were omitted in order to use them as negative controls. Subsequently, after the nuclear contrast (1:5000, blue of Hoescht-33342) and additional washes, the slide was mounted.

2.4. Confocal analysis, image acquisition and fluorescence quantification

All images were obtained with a Leica confocal laser scanning microscope (TCS SP5 DMI 6000CS, Leica Microsystems GmbH, Wetzlar, Germany) using a 40/60X oil immersion objective. FITC was excited at 488 nm and emission was detected between 510 and 550 nm. TRITC was excited at 568 nm and emission was detected between 585 and

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