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Class III β -tubulin in Normal and Cancer tissues

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Abstract

Microtubules are polymeric structures composed of tubulin subunits. Each subunit consists of a heterodimer of α - and β -tubulin. At least seven β -tubulin isotypes, or classes, have been identified in human cells, and constitutive isotype expression appears to be tissue specific. Class III β -tubulin (β III-tubulin) expression is normally confined to testes and tissues derived from neural crest. However, its expression can be induced in other tissues, both normal and neoplastic, subjected to a toxic microenvironment characterized by hypoxia and poor nutrient supply. In this review, we will summarize the mechanisms underlying β III-tubulin constitutive and induced expression. We will also illustrate its capacity to serve as a biomarker of neural commitment in normal tissues and as a pure prognostic biomarker in cancer patients.

Keywords

β III tubulin; *TUBB3*; biomarker; neural differentiation; cancer

β III-tubulin and the microtubules

Microtubules are filamentous, cytoskeletal polymers found in all eukaryotes and are involved in critical cellular processes such as mitosis, cell motility, and intracellular transport. The core cylinders of microtubules are composed of basic building blocks, α - and β -tubulin monomers, that spontaneously assemble to form functional subunits called heterodimers. These α/β heterodimers polymerize in a linear fashion to form “head-to tail” polar protofilaments with β -tubulin facing headwords and α -tubulin oriented toward the tails. Along with the head to tail interaction, heterodimers interact also laterally to form the cylinder that is the microtubule. Although the term “cytoskeletal” may imply stasis and permanence, microtubules are in fact highly dynamic and ethereal. The vibrant kinetics of microtubule assembly and disassembly are self-regulated at least in part by β -tubulins, which is not only a structural protein but a GTPase as well. As critical yet ever-changing components of cell function, microtubules are often targeted when designing novel therapeutics and pharmaceuticals [1].

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Multiple genes encode α - and β -tubulin isotypes. These genes show high homology [2] and conservation across vertebrate species [3] indicating origins from a common ancestral gene. Both α - and β -tubulin isotypes, like cytokeratin isotypes, demonstrate differential tissue expression patterns during embryogenesis, suggesting diversity in functionality and a specific role in the differentiation processes [4]. At least seven β -tubulin isotypes have been identified in the human genome [2]. These vary in the final 15 C-terminus residues which are exposed for the interaction with microtubule associated proteins (MAPs). They also differ in the composition of the 3'-UTR flanking region implicated in transcriptional and translational regulation. These modifications confer properties which match the metabolic and structural needs of a given tissue.

β III-tubulin is encoded by the *TUBB3* gene which firstly appeared in fish, both osteichthyes and chondrichthyes [3, 5], thus coinciding with the time of vertebrate evolution and exposure to higher tension of oxygen [6]. β III-tubulin is constitutively expressed in the central and peripheral nervous systems and in the testes, specifically in Sertoli cells.

In *in vitro* cultured cancer cells, constitutive *TUBB3* expression seems regulated in a cell-cycle dependent way, with maximal expression at the G₂/M phase of the cell cycle [7]. Such constitutive expression is linked to the expression levels of the RE1-silencing transcription factor (REST). REST binds to the first *TUBB3* exon and suppresses the constitutive *TUBB3* expression [8]. In other tissues, both normal and neoplastic, the expression of β III-tubulin is induced by exposure to a toxic microenvironment featured by hypoxia and poor nutrient supply.

In this review, we will describe the regulation of *TUBB3* in humans and its functional role in normal and diseased tissues. We will also provide a context for understanding why β III-tubulin has been extensively documented as a biomarker of poor outcome in a panel of solid malignancies.

Gene Structure of human *TUBB3*

The human *TUBB3* gene is located on chromosome 16q24.3 and consists of 4 exons that transcribe a protein of 450aa. A shorter isoform of 378aa derived from alternative splicing of exon 1 is devoid of part of the N-terminus and may be responsible for mitochondrial expression [9, 10], whose functional significance remains unknown. Similar to other β -tubulin isotypes, β III-tubulin has a GTPase domain which is essential in regulating microtubule dynamics [11].

Class I (the most represented and constitutively expressed isotype) and β III-tubulin differ by just 13aa within the 1–429aa region, whereas region 430–450aa is completely divergent. These variations in primary structure affect the paclitaxel binding domain [2, 12, 13] on β III-tubulin and may account for the ability of this isotype to confer resistance to paclitaxel initiated apoptosis [14]. Since paclitaxel is a Nur-77 mimic, β III-tubulin may represent the inherent survival pathway to Nur-77 induced cell death [14]. In fact, Nur-77 translocation

from nucleus to mitochondria activate a cell death program [15], which is inhibited by β III-tubulin expression [14].

Expression and function of β III-tubulin

A recent PUBMED search using keywords “class III β -tubulin” or “*TUBB3*” retrieved 610 references. Of these, 51% (309/610) relate to its role in neural differentiation. The knowledge of *TUBB3* as a neuronal marker date back to 1989 when the first monoclonal anti-class III β -tubulin antibody (TUJ1) became available [16]. The expression of β III-tubulin by medulloepithelial rosettes suggested that this isotype was one of the earliest markers to signal neuronal commitment in primitive human neuroepithelium. This hypothesis was later confirmed in other species [17, 18]. The critical importance of the *TUBB3* gene in neural development has also been confirmed by the study of *TUBB3* mutations in congenital syndromes. A panel of at least eight heterozygous missense mutations was shown to produce congenital fibrosis of extraocular muscles 3 (CFEOM3), a group of eye movement disorders caused by dysfunction of the oculomotor nerve [19]. The classical CFEOM3 symptoms, ptosis and restricted eye movements, are observed at birth. These symptoms are associated with additional nervous system disorders.

CFEOM3 patients exhibit peripheral axonal neuropathy, facial paralysis, and often intellectual and behavioral impairments. Conventional neuroimaging reveals a spectrum of abnormalities including hypoplasia of oculomotor nerves along with dysgenesis of the corpus callosum, anterior commissure, and corticospinal tracts. The commonest *TUBB3* mutation causing CFEOM3 results in a R262C amino acid substitution. A *TUBB3*R262C knock-in mouse model reveals axon guidance defects of the oculomotor nerve and central axon tracts without evidence of cortical cell migration abnormalities [19]. By contrast, six other diverse missense mutations in the *TUBB3* gene were observed in patients with cortical disorganization and axonal abnormalities associated with pontocerebellar hypoplasia but without ocular motility defects typical of CFEOM3 [20]. The diverse spectrum of phenotypic changes related to congenital *TUBB3* mutations highlights the pivotal role this protein plays in neuronal development. As further evidence of its specificity, *TUBB3* inactivation impairs progenitor proliferation which cannot be rescued or restored by other β -tubulins [21].

TUBB3 expression is also constitutively expressed in testis where it is regulated by androgen exposure during ontogenesis in mouse and rat Sertoli cells [22]. Hormonal influence of *TUBB3* expression has also been identified in neoplasia. Breast carcinoma cells, for example, show *TUBB3* under the control of estradiol via the estrogen receptor [23]. In prostate cancer, β III-tubulin expression is strongly associated with both TMPRSS2:ERG rearrangement, ERG expression and PTEN deletions, three key oncogenetic features of aggressive prostate cancer [24]. Also, colorectal cancer is more aggressive in young male patients in whom testosterone elevation and activation of *TUBB3* are connected with poor outcome [25, 26].

β III-tubulin and taxane-resistance: A need for critical revision?

The primary interest in β III-tubulin in oncology relates to its putative role in taxane-resistance. Large metanalyses [27] and a number of additional smaller studies [28–46] have clearly indicated that β III-tubulin is linked to poor outcome. Taxanes inhibit tubulin depolymerization thereby increasing intracellular levels of polymerized tubulin and arresting cellular functions. Early pivotal *in vitro* studies demonstrated that different proportions of tubulin isotypes affect microtubule dynamics. In particular, β III-tubulin was shown to enhance the rate of tubulin depolymerization [47], a feature that could account for resistance to taxanes, drugs that promote tubulin assembly. Indeed, a number of early studies of ovarian [48, 49] and lung [50] carcinomas supported this notion. However, this direct correlation was challenged by subsequent findings. For example, when β III-tubulin was moderately expressed under a conditional promoter, there was only weak induction (less than twofold) of paclitaxel-resistance [51]. Furthermore, increased expression of β III-tubulin was documented in patients treated with drugs acting as direct inhibitors of tubulin polymerization such as *Vinca* alkaloids [52]. Even more problematic, in breast cancer [53, 54], clear cell ovarian carcinoma [55] and melanoma [56], studies have reported that high expression of β III-tubulin was linked to sensitivity to taxane-based chemotherapy and not resistance. In this sense, in tissues where constitutive β III-tubulin is present, low expression of this tubulin isotype is a sign of de-differentiation and more aggressive disease. As an apparent paradox, in tissues where constitutive β III-tubulin is absent, its aberrant expression is associated in most cases, but not always, with an aggressive phenotype regardless if the disease is treated with a taxane or not [11, 56–61].

Postulating that β III-tubulin alone regulates microtubule dynamics and induces resistance to taxanes is clearly an over-simplification. In fact, many factors are capable to bind to β III-tubulin, such as γ -tubulin [62, 63] SEMA6A [64], GBP-1 [65] and Erp57 [10]. Moreover, *TUBB3* expression can occur in concert with other elements, when transcription is activated by exposure to a toxic microenvironment featured by low oxygen [66, 67] and poor nutrient supply [68]. In the 3' flanking region of *TUBB3*, there is an E-box (5'-RCGTG-3) which binds hypoxia induced transcription factors, HIF-1 α and -2 α . Accordingly, β III-tubulin staining appears in hypoxic areas within tumors [69]. Hypoxia triggers pro-survival pathways [70, 71] rendering cells resistant to taxanes along with a number of other drugs. In this context, β III-tubulin is not a passive bystander. In cell lines with an aggressive phenotype and constitutive β III-tubulin expression, β III-tubulin knockdown is associated with reduced growth and increased chemotherapy sensitivity when compared with control clones [72, 73]. β -tubulins are redox-controlled proteins. As such, they have cysteine residues that can be reversibly oxidized resulting in modification of protein function [74]. Khan and Luduena have proposed that oxidative stress and the thioredoxin system regulate the dimer/microtubule equilibrium [75]. Proteomic analysis has revealed that many factors that bind to β III-tubulin are involved in the oxidative stress and glucose deprivation response [10]. In structural terms, constitutive Class I (*TUBB*) and Class IVb (*TUBB2C*) β -tubulins contain a cysteine at position 239, while β III-tubulin has a cysteine at position 124. Position 239 can be readily oxidized while position 124 is relatively resistant to oxidation [5]. Thus, a relative abundance of β III-tubulin in situations of oxidative stress should enhance pro-

survival signals and cell integrity [65]. This concept appears especially plausible in light of the fact that, as mentioned above, β III-tubulin first appears in evolutionary history at the time of increased exposure to oxygen.

Among the components of the cytoskeletal gateway to resistance, a leading role is played by the GBP-1 (guanylate-binding protein 1) GTPase. This enzyme directly binds to β III-tubulin and facilitates the incorporation of a panel of pro-survival factors into the cytoskeleton including PIM-1 (Proviral Integration Site 1) and another 19 kinases [65]. Integrated analysis of GBP-1 along with upstream β III-tubulin regulators (HIF-1 α and -2 α , miR-200c) and downstream effectors of β III-tubulin enables accurate identification of a subset of cancer patients in whom the aggressive pathway is activated [65, 67, 76].

In functional terms, the expression of β III-tubulin in normal tissues seems to be finalized to the protection of the most precious progenitors in tissues. This phenomenon seems not specific of the neural progenitors [77–79]. In this sense, β III-tubulin could be considered as a marker of stemness. Also within cancer tissues, β III-tubulin expression may simply indicate an enrichment of cancer stem-like cells [80, 81], featured by certain ABC transporters such as ABCB1 (known as Pgp/MDR1), ABCG2 (known as BCRP) and very efficient systems of DNA repair. The coordinate expression of multiple factors aimed at the stem cell protection from xenobiotics may then cause the multidrug resistance phenotype responsible in patients for poor response to chemotherapy.

In summary, β III-tubulin is not a predictive biomarker of taxane-resistance. Instead, β III-tubulin is a pure prognostic biomarker only when its expression is conditioned by a toxic microenvironment. However, when its expression is driven by other factors, the β III-tubulin role is neutral or even opposite, acting as a marker of a more differentiated and less aggressive disease.

β III-tubulin as a drug target

Since the discovery of β III-tubulin's role in drug-resistance, a number of strategies have been pursued to inhibit its function. An early approach consisted of high throughput screening of cells over-expressing β III-tubulin with novel anti-tubulin agents. This strategy led to the synthesis of IDN5390 [12] and other seco-taxanes [82]. These drugs were designed *in silico* through computational modeling of the paclitaxel binding domain of β III-tubulin, which is slightly different from that of class I, the most expressed β -tubulin isotype. For the fact that β III-tubulin and the ABCB1 efflux pump are co-expressed in the same cancer stem-like cells, it is not surprising that drugs particularly active against β III-tubulin are not substrate of ABCB1 and produce cytotoxic effects in multidrug resistant cell lines [12, 82–84].

In silico modeling provided additional insights into potential mechanisms of β III-tubulin inhibition. Through this analysis, epothilones, particularly, patupilone (Epothilone-B), were proposed as promising agents, a promise that was borne out when patupilone-resistant ovarian and endometrial cancer cells exhibited significant down-regulation of *TUBB3* [84, 85]. Although patupilone is known to produce a dramatic therapeutic response in subset of

patients [86], a large multicenter ovarian cancer study of this agent failed to obtain its primary endpoint [87]. We suggest that the lack of inclusion of β III-tubulin expression as a criterion of enrollment contributed to this failure.

The *ex-novo* creation of novel β III-tubulin inhibitors is hampered by the absence of a simple, reliable *in vitro* test of β III-tubulin function. To be active, β III-tubulin requires dimerization, assembly in polymeric structures with additional cytoskeletal elements including MAP, and other post-translational changes which cannot be readily reproduced *in vitro*. An alternative strategy leverages the knowledge that β III-tubulin function is mediated through GBP-1. Unlike β III-tubulin, GBP-1 can be expressed *in vitro* in an active form and its downstream interactions with prosurvival kinases such as PIM-1 [65, 88] can be quantified in response to various compounds of interest. This approach led to the identification of aza-podophyllotoxin as a potential therapeutic inhibitor of β III-tubulin prosurvival function [89]. Noteworthy, podophyllotoxin is a known inhibitor of tubulin polymerization and competitive antagonist of the colchicine binding site [90, 91]. As demonstrated by the case of seco-taxanes, small changes to a natural pharmacophore may be an attractive avenue to generate compounds capable to inhibit the noxious β III-tubulin function.

Conclusion

β III-tubulin is the most investigated tubulin isotype. Its roles in neural development and oncology have warranted this intensive study. Recent analysis has demonstrated that β III-tubulin is part of a complex, pro-survival, molecular pathway activated by hypoxia and poor nutrient supply. Induction of this pathway in cancer is associated with an aggressive phenotype in the majority of patients, with some exceptions. In this sense, β III-tubulin is a pure prognostic biomarker whenever its association is linked to a more complex phenotype. On the other hand, β III-tubulin is not a predictive biomarker of taxane-resistance as originally thought, as its role in determining biological aggressiveness require other factors. Complete elucidation of β III-tubulin interactions, both upstream and downstream, may facilitate development of novel anti-cancer agents and ultimately transition β III-tubulin from a pure prognostic to an actionable predictive biomarker.

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Abbreviation list:

βIII-tubulin	Class III β -tubulin
MAP	microtubule associated proteins
ABC	ATP Binding Cassette
UTR	Untranslated Regione

CFEOM3	Congenital Fibrosis Of Extraocular Muscles 3
GBP-1	guanylate-binding protein 1
PIM-1	Proviral Integration Site 1
HIF-1α	Hypoxia Inducible Factor 1 α
HIF-2α	Hypoxia Inducible Factor 2 α

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Fig. 1:

Representative multiplexed fluorescent immunohistochemistry of a high grade serous ovarian cancer case stained with anti-TUBB3. In blue cells nuclei stained with DAPI; in green stromal cells stained with anti-vimentin; in yellow epithelial cancer cells stained with anti-cytokeratin; in pink TUBB3 expression stained with anti-TUBB3 (clone TUJ1). TUJ1 pattern of staining is cytoplasmic and as in this case is often heterogeneous within the cancer cell population in high grade serous ovarian.