

Deep Insight Section

Class III beta-tubulin, drug resistance and therapeutic approaches in cancers

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Abstract

Class III beta-tubulin is one of the critical proteins associated with microtubule assembly, important to many cellular functions including mitochondrial respiration and intracellular trafficking. Widely regarded as a specific neuronal marker in developmental neurobiology and stem cell research, it is also highly expressed in a wide range of tumors of both neuronal and non-neuronal origin. The expression of class III beta-tubulin is tightly controlled at multiple levels with tissue-dependent mechanisms of regulation. For instance, class III beta-tubulin expression is under the control of estrogens in breast cancer cells but is influenced by exposure to hypoxia and poor-nutrient supply in ovarian cancer. In some but not all cancers, class III beta-tubulin expression is purely a prognostic biomarker, predicting poor outcome of patients regardless of chemotherapy treatment. Moreover, the expression of class III beta-tubulin does not confer an aggressive phenotype by itself. Instead, class III beta-tubulin functions like a cytoskeletal gateway, which enhances the incorporation of pro-survival kinases into the cytoskeleton and protects them from degradation. The associations of class III beta-tubulin with survival kinase PIM-1, RNA-binding protein HuR, microRNAs are examples highlighting the functional complexity of this protein. The utility of class III beta-tubulin as a prognostic biomarker can also greatly improve if combined with these pro-survival partners. Subsequently, pharmacogenetic approaches, designed to counteract and target these pathways and associated-factors concurrently, might lead to better therapies and prognostic tools for class III beta-tubulin expressing cancers.

1. Introduction

Microtubules (MTs) are highly dynamic, cytoskeletal structures that are essential to a variety of cellular functions, including cell division, proliferation, migration, protein trafficking, intracellular transport, and maintaining cellular polarity (Kreis and Vale, 1993; Katsetos and Dráber, 2012). Microtubules are formed by the polymerization of the heterodimers: α -tubulin subunits and β -tubulin subunits, the isotypes of which are encoded by multiple genes (Kreis and Vale, 1993; Katsetos and Dráber, 2012). Interactions with several microtubule associated proteins (MAPs) are crucial to diverse microtubule functionality. The phylogenetic analyses from vertebrate species suggest α and β isotypes of

microtubules are highly conserved among species but have complex expression pattern, which denote cellular and functional specificity and diversity (Katsetos et al., 2003).

There are at least seven mammalian beta-tubulin isotypes known to have formed through distinct gene products without having gone any splicing events (Ludueña, 1998).

These beta-tubulin subtypes differ from one another mainly in a region, which is limited to the last 15 C-terminal residues.

Class III beta-tubulin differs from other β -tubulin subtypes in its post-translation changes, such as differences in phosphorylation and polyglutamination in the same terminal residues (Orr et al., 2003). Furthermore, TUBB3 gene is the most conserved subtype across vertebrate species

(Katsetos et al., 2003). In somatic tissues, class III beta-tubulin has mostly been used as a biomarker for neural stem cells and neuronal differentiation during fetal and post-natal development (Caccamo et al., 1989; Verdier-Pinard et al., 2009). It is also constitutively expressed in the Sertoli cells of the testes, transiently expressed in fetal respiratory epithelium, and at lower levels at other tissues (Verdier-Pinard et al., 2009; Abel et al., 2009). In cancers however, class III beta-tubulin is expressed in tumors of both neuronal and non-neuronal origin. Class III beta-tubulin is by far the most investigated microtubule isotype in human cancer. A growing body of evidence suggests high expression of class III beta-tubulin is associated with poor prognosis and aggressiveness of several cancers including non-small cell lung cancer (NSCLC), breast, ovarian, and gastric cancers (Katsetos and Dr ber, 2012; Katsetos et al., 2003). However, there are also reports of expression of class III beta-tubulin and good outcome as reported in clear cell ovarian cancer and estrogen-receptor negative breast cancer (Aoki et al., 2009; Wang et al., 2013). This report will try to highlight the function of class III beta-tubulin and explain the seemingly contradicting therapeutic results.

2. Microtubule targeting agents - growing list of agents for cancer therapeutics

The dynamic reorganization of the microtubules allows for the formation of mitotic spindle, which is crucial for the faithful segregation of chromosomes into daughter cells during cell division (Stanton et al., 2011).

Thus, disrupting microtubules to inhibit mitotic cell division has become an attractive pharmaceutical approach to treat many different cancers. Accordingly, Microtubule Targeting Agents (MTAs) are some of the most widely used drugs in cancer treatment and have met significant clinical success (Jordan and Wilson, 2004). MTAs are natural, small molecules that interfere with microtubule function at low concentrations by preventing the formation of normal mitotic spindle during cell division, a process that goes awry during cancer development (Jordan and Kamath, 2007). Classically, MTAs being used in cancer treatment fall into two kinds of drugs - MT stabilizing (taxanes and epothilones) and MT destabilizing (*Vinca* alkaloids) agents. Taxanes - paclitaxel docetaxel, abraxane, and cabazitaxel, are usually administered in the treatment of a wide range of solid cancers, such as breast, ovarian, NSCLC, and cancers of the head and the neck. *Vinca* alkaloids, such as vinorelbine, vinflunine and vinblastine, are usually used in the treatment of hematological malignancies like the lymphoma and leukemia (Jordan and Wilson, 2004; Morris and

Fornier, 2008). Both classes of drugs target specific binding sites of beta-tubulin, which disrupt the microtubules, inhibiting its assembly or disassembly, leading to cell death mostly by apoptosis (Morris and Fornier, 2008). However, the exact mechanism by which each drug works to inhibit microtubules is still speculative and not fully explained. Taxanes are regularly administered as part of adjuvant therapy in patients with breast, ovarian, and non-small cell lung cancer, and recent indications suggest that it can further improve prognosis and therapy through combination with biologic agents (Dumontet and Jordan, 2010; Karki et al., 2014).

The clinical success of taxanes led to development of drugs like epothilones, which are macrolide antibiotics and can enhance microtubule polymerization and structurally more suitable to synthetic modifications (Morris and Fornier, 2008; Dumontet and Jordan, 2010). Similar to taxanes that bind to beta-tubulin, epothilones exert similarly and are thought to compete with taxane binding sites. More importantly, early trials of epothilones seem to indicate that they have better efficacy in patients resistant to taxane-including regimen (Cheng et al., 2008; Goodin et al., 2004). Some of the modified versions of epothilones include patupilone, a naturally occurring epothilone, which was found to be 20 times more potent than paclitaxel in taxane-resistant cell lines. Ixabepilone, a semisynthetic version, has also shown to be effective in resistant cell lines. Yet, many promising agents that interfere with microtubules like epothilone B and D analogues, colchicines, and laulimalide binding agents are also currently at different stages of development (Morris and Fornier, 2008; Dumontet and Jordan, 2010).

3. Mechanism of drug resistance - the role of beta III tubulin

Efficacy of taxanes and other MTAs is limiting due to the development of acquired and intrinsic resistance of tumor cells to the drugs, as well as increased hypersensitivity and neurological toxicities. The mechanistic details of such chemoresistance are entirely not clear at present. However, earlier studies in the 90s on class III beta-tubulin suggested that this protein mediated chemoresistance in response to taxanes and widely regarded to be predictive biomarker to taxane-based therapies (Derry et al., 1997). Owing to different mechanisms of action, this classical theory entailed class III beta-tubulin to be predictive of taxane based chemotherapy but not of *Vinca* alkaloids. Initial studies on this protein supported this theory, where high expression of class III beta-tubulin was associated with chemo-resistance in taxane-based therapies in different cell-based models.

This positive correlation of chemo-resistance

association was also seen by our group when we investigated class III beta-tubulin in the context of using paclitaxel in ovarian cancer (Kavallaris et al., 1997; Mozzetti et al., 2005). More recent data on class III beta-tubulin and various MTA agents have been rather conflicting. In one of the studies, class III beta-tubulin gene silencing desensitized effects of both taxanes as well as other drugs, in particular cisplatin. In another study, epothilones were found to be extremely active in ovarian cancer cell models and other gynecological cancers but not for lung cancer, even when both sets of cancers exhibited high class III beta-tubulin content (Gan et al., 2007; Mozzetti et al., 2008; Carrara et al., 2012). To further gain insight into this contradiction, our group recently did a comprehensive review of 59 translational studies assessing class III beta-tubulin in many different types of cancers. Not surprisingly, our analysis refuted the notion that class III beta-tubulin is predictive of taxane-based chemotherapy (Karki et al., 2013). In those studies that claimed class III beta-tubulin to be predictive of response to MTA-including chemotherapy, either the sample size was small or analyses were not stringent. Instead, our analysis of the studies show that class III beta-tubulin is a pure prognostic biomarker in some solid malignancies, regardless of the choice of chemotherapies employed (Karki et al., 2013). In order to understand the basis of the prognostic capability of class III beta-tubulin, it is necessary to understand the mechanisms regulating its expression and the varied functions this protein may have in the context of different cells and tissues (Karki et al., 2013).

4. Regulation and expression of class III beta-tubulin - hormones and gender

In cases of ovarian and breast cancers, hormones may play a significant role in the expression and regulation of class III beta-tubulin. In breast cancer, class III beta-tubulin expression is under the control of estrogens. This was demonstrated in a study where estradiol exposure to MCF-7 breast cancer cell line, positive for estrogen receptor (ER), upregulated the TUBB3 mRNA and class III beta-tubulin protein. However, in cells negative for ER such as MDA-MB-231, no such induction was observed (Saussede-Aim et al., 2009). In this context of ER positive cells, expression of class III beta-tubulin cannot confer an aggressive phenotype and be linked to resistance of the drug. Instead, these ER positive patients with high class III beta-tubulin expression have better outcome because they are sensitive to aromatase inhibitors and other anti-hormonal therapies. This was further confirmed in this study using ER modulators: tamoxifen - a selective ER modulator and fulvestrant - a pure antagonist of ER. These

hormonal modulators were able to inhibit class III beta-tubulin mRNA induction suggesting class III beta-tubulin expression is mediated through ER dependent pathway (Saussede-Aim et al., 2009). The evaluation of class III beta-tubulin to predict responses and outcome in triple-negative breast cancer would be interesting as this breast cancer is devoid of effect by ER and other hormones. A recent study in *Lancet* has demonstrated that exposure to high levels of oestradiol and progesterone found in breast and ovarian cancer patients are linked to *BRCA1/BRCA2* mutations (Widschwendter et al., 2013). In a recent translation study of ER negative breast cancer, class III beta-tubulin was found to correlate with good pathogenic response to chemotherapy, suggesting the protein maybe identified differently in specific subsets of breast cancer (Wang et al., 2013). It is unclear if there is any relationship between *BRCA1/BRCA2* mutations, chemotherapy sensitivity, and class III beta-tubulin expression. Hormones and gender can also play a role in expression of class III beta-tubulin in male cancers. In a panel of 23 colorectal cancer cell lines, the expression of class III beta-tubulin is increased in response to androgens only in males. This basal activation of class III beta-tubulin seems associated with poor clinical outcome in male colorectal cancer patients (Mariani et al., 2012). It was further found that CYP17A1, a critical enzyme regulating androgen levels, have a specific allele (GG phenotype) that confers colorectal patients with high level of androgens (Mariani et al., 2012). In prostate cancer, there is also an aspect of class III beta-tubulin regulation by androgen levels. In one study, class III beta-tubulin increased significantly in castration-resistant prostate cancer (CRPC) patients after treatment with anti-androgen therapy. This is probably due to loss of regulation by androgen receptor or CRPC association with hypoxic conditions (Forde et al., 2012).

5. Regulation of beta III tubulin - exposure to microenvironment and survival pathway

In many cancers, class III beta-tubulin is a part of an adaptive function circuit, which allows cancer cells thrive in microenvironment featured with poor oxygen and low nutrient supply. Our previous work has demonstrated that hypoxia is able to induce TUBB3 gene expression through HIF1 binding to its 3' region (Raspaglio et al., 2008). In ovarian cancer the regulation and expression of class III beta-tubulin is more complex and involves the role of additional transcription factors, RNA-binding proteins such as HuR, microRNAs, and components of the survival pathway involved in the adaptation to hypoxia. In hypoxic and hypoglycemic conditions, prevalent in many advanced cancers

including ovarian cancer, RNA binding protein like HuR facilitates class III beta-tubulin translation to counteract glucose shortage (Raspaglio et al., 2010). HuR expression is nuclear in normal tissues but is found to be cytoplasmic in advanced cancers exposed to hypoxia. Cytoplasmic HuR and high class III beta-tubulin in ovarian cancer patients are indicators of exposure to hypoxia and are linked to poor outcome (Raspaglio et al., 2010). A more recent work from our group and others have further elucidated a combined regulatory mechanism that drives class III beta-tubulin expression through HuR and miR-200C in ovarian cancer (Prislei et al., 2013). When HuR is nuclear, a condition typical of low stage ovarian cancer, high expression of miR-200C inhibits TUBB3 expression and results in good prognosis. On the other hand, when miR-200C is associated to cytoplasmic HuR, the conversion of TUBB3 mRNA into beta III tubulin is enhanced, resulting in poor outcome of patients (Prislei et al., 2013). This report demonstrates that the same microRNA can exert different cellular functions for class III beta-tubulin based on location-specific interaction with the RNA-binding protein HuR.

In ovarian cancer, class III beta-tubulin function is linked to the adaptation to hypoxia and poor nutrient supply by incorporating pro-survival kinases like PIM1 into the cytoskeleton. The preferential incorporation of PIM1 into microtubules facilitates the cytoskeletal increase of the GBP1 GTPase. GBP1 incorporation stabilizes binding of the PIM1 kinase into microtubules and protects PIM1 from rapid degradation. This mechanism is not specific only for PIM1 but it is shared by additional kinases such as NEK6, AXL, and others (De Donato et al., 2012). As an ensuing mechanism, signaling of these pro-survival pathways is prolonged, thus enabling cancer cells to thrive in hypoxic conditions. Such analogous adaptation of cancer cells in harsh microenvironment is seen in many cancers, but the role of cytoskeleton in the process is only beginning to emerge with recent data from class III beta-tubulin expression. In some ovarian cancer cells, regulation of class III beta-tubulin is under the control of a transcription factor, Gli1, one of the drivers of epithelial-to-mesenchymal transition program and also a hallmark of metastatic potential of solid tumors. In this study, Gli1 was able to increase class III beta-tubulin expression under hypoxic conditions conferring a more invasive ovarian cancer phenotype (Mozzetti et al., 2012).

6. Utility of combined biomarkers with beta III tubulin to increase prognostics

From both our recent review of translational studies of cancers and previous studies on class III beta-tubulin, it is clear that the utility of class III beta-tubulin as a biomarker increases significantly when used in combination with additional biomarkers, proteins, or other discrete factors in different cancers. In fact, it is important to stress the concept that class III beta-tubulin does not mediate the resistant phenotype alone but only in a multi-molecular concerted pathway. Therefore, it is not surprising that this protein, when taken as a single agent, can even be a hallmark of good outcome, as seen in estrogen positive breast cancer. In a study conducted in gastric cancer, the combination of thymidine phosphorylase (TP)-positive and class III beta-tubulin negative tumors gave a stronger predictive power than TP negative and class III beta-tubulin positive tumors alone. Both overall survival (OS) and progression free survival (PFS) reached significance when surviving class III beta-tubulin was analyzed in combination (Gao et al., 2011). In another study in gastric cancer, inactivation of Bca1 and low class III beta-tubulin provide a better utility in predicting responses to cytotoxic therapy, regardless of taxane-containing or taxane-free drug combinations (Moiseyenko et al., 2013). In breast cancer, the double-negative expression of class III beta-tubulin and survivin responded significantly better to docetaxel and had longer PFS ($p < 0.05$) when compared with double-positive patients (Yuan et al., 2012). In another clinical set of breast cancer, the class III beta-tubulin negative tumors when combined with the BCL-2 and ERCC1 proteins increased predictive potential in response to CP (carboplatin/paclitaxel) therapy (Chen et al., 2012). Since BCL-2 and Survivin are both important in the pathway that regulates apoptosis; they could possibly impact sensitivity to chemotherapy. In ovarian cancer, high expression of both class III beta-tubulin and PIM1 combination has a synergistically higher prognostic potential (De Donato et al., 2012). In thymic epithelial cancer patients, ERCC1, BRCA1, and class III beta-tubulin combination strongly correlate with one another and can be used to improve accuracy of prognostic and predictive tests against chemotherapeutic regimens (Kaira et al., 2011). In lung cancer, several studies support the notion of improved efficacy of integrated biomarkers. In

completely resected patients with NSCLC, double negative patients with class III beta-tubulin and ERCC1 had even more significant improved outcome ($p=0.023$) than when they were used individually (Okuda et al., 2008). In patients relapsed and treated with platinum/taxane, double negative ERCC1 and class III beta-tubulin tumors predicted OS significantly. The prediction was not significant when class III beta-tubulin was used alone ($p=0.015$ vs. 0.087) (Azuma et al., 2009).

7. Therapeutic approaches with class III beta-tubulin

Microtubules enriched of class III beta-tubulin are featured by increased sensitivity to epothilones, particularly to epothilone B. The configuration of the pocket binding epothilone B (patupilone) in class III beta-tubulin differs from the one present in class I beta-tubulin, the most abundantly expressed beta-tubulin (Magnani et al., 2006; Ferlini et al., 2005). Therefore, cells with high expression of class III beta-tubulin appears more sensitive to patupilone (Mozzetti et al., 2008). This property explains the increased effects of epothilones noticed in a large number of clinical trials conducted in patients relapsing after multiple lines of chemotherapy (Ferrandina et al., 2012). Therefore, it seems rather attractive to use class III beta-tubulin as a potential biomarker for selection of patients eligible for treatment with epothilones. In this regard, such approach necessitates development of an integrated biomarker as mentioned above, to ensure active status of class III beta-tubulin function.

Based on mechanism of regulation and interaction with its functional partners in the tumor microenvironment, class III beta-tubulin can be also exploited to develop cancer therapeutics. The rapid evolution of technologies is providing incredible and unprecedented amount of information about various forms of cancers. It is becoming feasible to dissect functional pathways in cancer cells that are responsible for biologic features, in particular, the mechanisms leading to resistance of cancer cells to cell death programs and ability to resist to radio- and chemotherapy. Disruption of such pathways, in a "targeted" fashion, should represent the next frontier in the development of innovative targeted anticancer drugs. In this context, the screening of new molecules to directly inhibit class III beta-tubulin could be challenging because of limitations of current technology to synthesize active version of the protein. In fact, the functional active tubulin not only requires dimerization but also posttranslational changes and incorporation into microtubules. On the other hand, downstream effectors of class III beta-tubulin do not have such limitations. The proteins, GBP1 and PIM1, for example, can be expressed as recombinant active proteins. Their protein-protein interactions may be

useful to identify new scaffolds to generate novel therapeutics that are active in drug-resistant cells. Therefore, such an inhibitor of the GBP1:PIM1 interaction might be useful in those patients, who exhibit high level of class III beta-tubulin and consequently, have a poor response to chemotherapy. As opposed to the currently available targeted agents capable of inhibiting one kinase family, this new agent is expected to inhibit the incorporation of a wide number of kinases into the cytoskeleton. Such an effect will have a broader but telling impact on the resistant cancer phenotype than that achieved by inhibiting a single kinase family.

8. Summary and conclusion

Class III beta-tubulin will continue to be at the forefront of cancer therapeutics, owing to its expression in the majority of solid tumors and its prognostic potential.

The field is shifting away from the hypothesis linking class III beta-tubulin directly to resistance to MTAs based on a single protein mechanism.

The better comprehension of the mechanisms underlying class III beta-tubulin function is improving its potential use as a prognostic biomarker and as a potential factor to select alternative treatments for those patients, where class III beta-tubulin pathway confers an aggressive phenotype.

Its role in the functional gateway of cytoskeletal drug resistance suggests that inhibition of this protein by novel targeted agents will be useful in the treatment of diseases, which are currently refractory to standard treatments. For all these reasons, we expect class III beta-tubulin will continue to be actively pursued as a source of new diagnostics and therapeutics for aggressive cancers.

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