https://doi.org/10.31925/farmacia.2018.4.12 **ORIGINAL ARTICLE**

TUBULIN, A POSSIBLE MARKER FOR THE PROGNOSTIC STRATIFICATION AND THERAPY IN PAPILLARY THYROID CARCINOMA

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Manuscript received: April 2018

Abstract

The present study aims to analyse the expression of class III β-tubulin (TUBB3) in different histological subtypes of papillary thyroid carcinoma (PTC), its relationship with the clinico-pathological factors and the potential prognostic role in the risk stratification and therapy. We evaluated the immunohistochemical TUBB3 expression in 70 cases of sporadic PTC divided in low- and high-risk subgroups based on histological criteria. We demonstrated a significant correlation between TUBB3 expression (low and moderate *versus* high) and the risk subgroups and tumour relapse, respectively. No association was found between TUBB3 expression and age, gender, tumour size, tumour focality, lympho-vascular invasion, extrathyroidal extension, lymph node metastases and tumour stage. Our results sustain the potential of TUBB3 as marker for prognostic stratification. Consequently, the therapy with taxanes, anti-microtubule agents that inhibit mitosis by disrupting microtubules, could be considered in the aggressive PTC cases that need a personalized therapy.

Rezumat

Scopul prezentului studiu este de a analiza expresia β-tubulin III (TUBB3) în diferite subtipuri histologice de carcinom tiroidian papilar (CTP), relația TUBB3 cu factorii clinicopatologici și potențialul rol prognostic în evaluarea riscului și terapie. Expresia imunohistochimică a TUBB3 a fost evaluată în 70 cazuri de CTP sporadic, împărțite în două subgrupuri în funcție de criterii histologice, cu risc scăzut și cu risc înalt. A fost demonstrată o corelație semnificativă între expresia TUBB3 (scăzută și moderată *versus* înaltă) și subgrupurile de risc, respectiv recidiva tumorală. Nu au existat diferențe semnificative statistic între expresia TUBB3 și vârstă, sex, dimensiune tumorală, focalitate, invazie limfo-vaculară, extensie extratiroidiană, metastază nodală și stadiu tumoral. Rezultatele obținute susțin potențialul TUBB3 ca marker prognostic pentru stratificare. Consecutiv, terapia cu taxani, agenți anti-microtubulari care inhibă mitoza prin dezasamblarea microtubulilor, ar putea fi luată în considerare pentru cazurile de CTP agresiv care necesită o terapie personalizată.

Keywords: tubulin, papillary thyroid carcinoma, risk stratification, therapy

Introduction

Thyroid carcinomas are the most frequent endocrine tumours, with a frequency of 1 - 3% of malignant tumours, and a higher incidence in females than in males [32, 40, 42]. World Health Organization (WHO) classifies thyroid carcinomas into four categories: papillary, follicular, medullary and anaplastic [42]. Papillary thyroid carcinoma (PTC) accounts for 70 - 85% of all thyroid carcinomas and hence it is considered the most common type [62].

PTC incidence is exponentially increasing world-wide, fact which can be explained by deeper diagnostic experience associating high-resolution medical imaging, fine needle aspiration biopsy, pathological, immune-histochemical and molecular exams [54, 81]. PTC prognostic is generally favourable, with long-term life expectancy [40, 42]. Nevertheless, there are cases where the lymph node or distant metastases occur rapidly and the survival is poor [40, 41, 61, 67, 77]. To identify the cases with poor prognostic is crucial at the time of diagnosis. Therefore, new prognostic factors should be confirmed to allow a prognostic stratification and, implicitly, a different therapeutic management [68].

Not less than 15 histological variants of PTC are described: conventional, follicular, oncocytic, with columnar cells, tall cells or clear cell, diffuse sclerosing, solid, cribriform, macrofollicular, hobnail, with stroma "fasciitis-like", microcarcinoma, mixed and dediffentiated forms [40, 41]. Some of these histological forms - namely the solid tumours, tumours with tall or cylindrical cells, "hobnail" variant, the angioinvasive follicular papillary carcinomas, or those that associate

poorly differentiated areas, squamous or anaplastic, seem to have a poorer prognostic [26, 27, 40].

The possibility for certain histological variants of PTC to be defined as high-risk or low-risk for an unfavourable outcome is highly debated. This classification can open perspectives for an individualized, more aggressive therapy, applied to the cases with poor prognostic, compared to the classical treatment of PTC [26, 27, 72].

On the other hand, a large gallery of molecular markers is analysed, following their validity as prognostic factors for PTC [68]. Unfortunately, despite the large number of studies focused on this topic, the results are still controversial. Consequently, the research interest is continuously enlarged, new candidates being added in the molecular gallery. One of these molecules is tubulin.

Microtubules are cytoskeletal proteins, made of two categories of α and β tubulin heterodimers, with multiple *isotypes* and with a varied, specific composition compared to the type of tissue and the intra-cellular functions. Microtubules contribute to maintaining the cellular form and also to the intracellular transport and chromosomal segregation during mitosis, with the consecutive formation of the mitotic spindle [29, 52]. Multiple studies proved the implication of class III of β-tubulin (TUBB3) in the malignant cell transformation and the appearance of carcinomas. Higher levels of TUBB3 are reported in brain, lung, colorectal, ovarian, prostate and laryngeal carcinomas [6, 15, 21, 29, 33, 35, 78, 84, 86]. The analysis of TUBB3 expression in relation to therapy showed differences in response and resistance to treatment. TUBB3 overexpression is associated to a poor prognostic and, frequently, to chemoresistance to the neo-adjuvant therapy with taxanes of neo-adjuvant chemotherapy with a role in microtubules stabilization [43], used in lung, uterus, ovary, colon or breast cancer [14, 44, 69, 76].

Strictly referring to the thyroid tumour pathology, literature review indicates few papers that analyse TUBB3 in anaplastic cell thyroid carcinoma and the treatment with anti-microtubule agents that inhibit mitosis by disrupting microtubules [1, 18]. To the best of our knowledge, there are only two studies that focuses on TUBB3 expression in PTC [10, 11].

Within this context, our study analyses the TUBB3 expression in different histological subtypes of PTC and its relationship with the classical clinico-pathological factors, aiming to bring evidences for its potential prognostic role in the risk stratification and, possible, in therapy.

Materials and Methods

Patients

We analysed 70 cases of sporadic papillary thyroid carcinoma diagnosed at the Pathology Department of "Sf. Spiridon" Emergency County Hospital, Iași, Romania, from 2010 to 2016. The research was approved by the Ethics Committee of "Grigore T. Popa" University of Medicine and Pharmacy in Iași, Romania, pursuant to the ethical standards of Helsinki declaration regarding the patients' informed consent to the use of biologic material for scientific purpose. All the patients underwent total thyroidectomy, and in 41 cases regional lymphadenectomy was also performed. The patients' clinico-pathological characteristics (age, gender, tumour size, and lymph node metastases) were obtained from the medical files.

The cases were reassessed by three pathologists who agreed on the histological variant of PTC, multifocality, lympho-vascular invasion and the extrathyroidal extension (tumour cells present into perithyroidal soft tissues, beyond the thyroid capsule). They also chose the appropriate paraffin-embedded tissue fragment for the immunohistochemical exam. Based on the histological variants of PTC, the study group was divided into two subgroups, namely low- and high-risk. The low-risk subgroup included conventional and follicular variants, characterized by indolent behaviour and favourable prognosis [26, 27, 40]. The high-risk group included columnar cell, tall cell, cribriform, hobnail, diffuse sclerosing, solid, angioinvasive follicular, conventional with dedifferentiation to squamous cell carcinoma and oncocytic with undifferentiated solid areas variants, known as aggressive subtypes in clinical course and poorer prognosis [26, 27, 40].

Immunohistochemistry

Tissue sections as thick as 4 μm were cut from paraffin blocks and disposed on slides coated with poli-L-lysine to ensure a better adhesion of the tissue. The slides were then dewaxed in xylene and rehydrated in four baths of ethanol with decreasing concentrations (100%, 90%, 80% and 70%). In order to unmask the antigen, it was used the Heat-Induced Epitope Retrieval (HIER) technique, by immersing the slides in pH 6 citrate buffer and heated in a water bath at 98°C for 30 minutes. The slides were left to cool at room temperature, rinsed in distilled water and incubated with hydrogen peroxide 3% for 10 minutes to block the endogenous peroxidases. Afterward, the primary antibody for β/beta-tubulin 3 (rabbit polyclonal, Thermo Scientific, USA), at a dilution 1:200, was applied, and the slides were incubated overnight at 4°C. The amplification of the immune reaction was made by using appropriate secondary and tertiary antibodies of the UltraVision Quanto Detection System HRP DAB (Thermo Scientific, USA). The reaction was developed with 3.3'-diaminobenzidine tetrahydrochloride chromogen (Thermo Scientific, USA). The sections were counterstained with Lillie's modified Haematoxylin. The correctness of the technique was

simultaneously checked, running both positive and negative controls.

Semi-quantitative assessment

For the assessment of TUBB3 it was used the score proposed for small cell lung carcinoma [57], based on the percentage of positive tumour cells and the intensity of the immune-reaction, as follows: high expression for > 50% positive tumour cells and strong intensity of immune-reaction (3+), moderate expression for > 50% positive tumour cells and moderate immune-intensity (2+), and low expression for ≤ 50% positive tumour cells and/or moderate or low immunostaining (≤ 2+).

Statistical analyses

The data were analysed using the Chi-square test (Maximum-Likelihood, Yates and Mantel-Haenszel) (SPSS V.19 - SPSS Inc., IBM Corporation, Chicago, IL, USA). Statistical significance was considered for $p < 0.05$.

Results and Discussion

Clinico-pathological outline

The study group comprised 53 (75.72%) women and 17 men (24.28%). The age at diagnosis ranged between 17 and 86 years old with a mean of 49.27 ± 14.74 years old. In the entire group, 44 patients (62.86%) were under 55 and 26 patients (37.14%) were over 55 years old at the time of diagnosis. The tumour size largely varied, ranging from 11 mm to 100 mm, with a mean of 32.32 ± 21.32 mm.

The low-risk subgroup comprised 23 cases (32.86%) of PTC, out of which 16 cases (22.86%) were diagnosed as conventional subtype and 7 cases (10%), as follicular subtype. The high-risk subgroup included 47 cases (67.14%), with the following distribution of the histological variants: columnar cell - 3 cases (4.28%), tall cell - 15 cases (21.43%), cribriform - 6 cases (8.57%), hobnail - 1 case (1.43%), diffuse sclerosing - 5 cases (7.14%), solid - 6 cases (8.57%), angioinvasive follicular - 7 cases (10%), conventional with dedifferentiation to squamous cell carcinoma - 3 cases (4.28%), oncocytic with undifferentiated solid areas - 1 case (1.43%).

Multifocality was present in 34 cases (48.57%), extra-thyroidal extension in 55 cases (78.57%), and lympho-vascular invasion in 47 cases (67.14%). Lymph node metastases were found in 24 cases (47.05%) - N1a in 13 patients and N1b in 11 patients. According to the WHO guidelines, the Classification of Malignant Tumours (TNM) and the College of American Pathologists Clinical Guidelines, the cases were classified into primary tumour (pT) categories, as follows: pT1 - 4 cases (5.72%), pT2 - 6 cases (8.57%), pT3 - 54 cases (77.14%) and pT4 - 6 cases (8.57%).

Eight patients relapsed, 6 of them were in the high-risk subgroup (diffuse sclerosing type - 2 cases, solid type - 2 cases, angioinvasive follicular type - 1 case, conventional with dedifferentiation to squamous cell carcinoma type - 1 case), and the other two in the low-risk subgroup (conventional variant).

Assessment of β-tubulin 3 expression

TUBB3 low expression was noted in 3 cases with low intensity of cytoplasmic staining and focal location in less than 50% of the tumour cells (Figure 1).

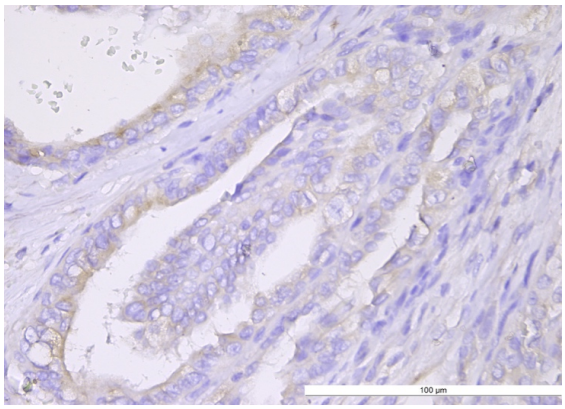

Figure 1.
Focal cytoplasmic immunoreaction of low intensity - PTC, conventional subtype (IHC anti-TUBB3, ×400)

A moderate expression was reported in 18 cases, where the cytoplasmic staining intensity was moderate in over 50% of tumour cells, present either focal or a diffuse pattern in the specimens.

The high expression was assessed in 49 cases; here, the immunoreaction had a strong and homogeneous intensity in almost 100% of the tumour cells (Figure 2).

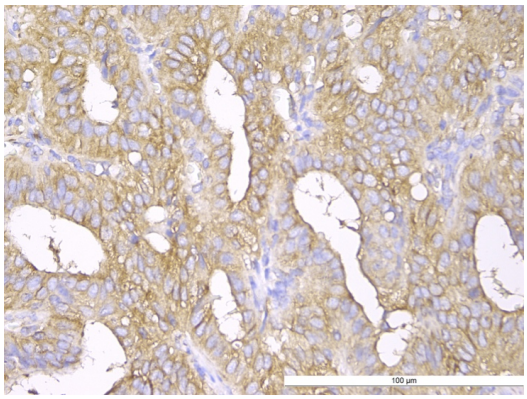

Figure 2.
Diffuse cytoplasmic immunoreaction of strong intensity - follicular subtype (IHC anti-TUBB3, ×400)

In the adjacent benign thyroid tissue, TUBB3 was negative in all the cases with low and moderate TUBB3 expression in tumour cells, and in 43 out of 49 cases with high expression. Moreover, all relapse cases had negative TUBB3 expression.

The correspondence between TUBB3 expression and the clinico-pathological characteristics, including the risk subgroups, was summarized in Table I.

Table I

Relationship between TUBB3 expression and clinico-pathological characteristics

Clinico-pathological characteristics	TUBB-3 expression		Chi-square test
	low and moderate (n = 21)	High (n = 49)	
Age at diagnosis			
< 55 years old	12 (27.3%)	32 (72.7%)	p = 0.517
≥ 55 years old	9 (34.6%)	17 (65.4%)	
Sex			
male	3 (17.6%)	14 (82.4%)	p = 0.201
female	18 (34%)	35 (66%)	
Tumour size (median)			
< 25 mm	8 (25%)	24 (75%)	p = 0.402
≥ 25 mm	13 (34.2%)	25 (65.8%)	
Histopathologic subtype			
low risk group	11 (47.8%)	12 (52.2%)	p = 0.023
high risk group	10 (21.3%)	37 (78.7%)	
Focality of the tumour			
unifocal	8 (22.2%)	28 (77.8%)	p = 0.144
multifocal	13 (38.2%)	21 (61.8%)	
Lympho-vascular invasion			
absent	4 (17.4%)	19 (82.6%)	p = 0.107
present	17 (36.2%)	30 (63.8%)	
Extrathyroidal extension			
absent	3 (20%)	12 (80%)	p = 0.340
present	18 (32.7%)	37 (67.3%)	
Tumour stage			
T1 + T2	1 (10%)	9 (90%)	p = 0.136
T3 + T4	20 (33.3%)	40 (66.7%)	
Lymph node metastases			
N0	7 (41.2%)	10 (58.8%)	p = 0.158
N1	5 (20.8%)	19 (79.2%)	
Tumour relapse			
absent	16 (25.8%)	46 (74.2%)	p = 0.033
present	5 (62.5%)	3 (37.5%)	

Correlations between β-tubulin 3 expression and clinico-pathological prognostic factors

Statistical analysis revealed no correlation between TUBB3 expression (low and moderate *versus* high) and age, gender, tumour size, tumour focality, lympho-vascular invasion, extrathyroidal extension, lymph node metastases and tumour stage. Our results showed significant differences between TUBB3 expression and histological subtypes, defined as risk subgroups, and tumour relapse (Table I).

Expression of β-tubulin 3 in normal status and malignancies

TUBB3 is usually identified in cells of neuronal origin, where it contributes to the formation of dynamic microtubules which are essential in neurite growth and maintenance [29, 30, 36]. TUBB3 is also expressed in other normal tissues – namely the testicle, small intestine and placenta [35].

The interest in the study of TUBB3 is justified by the part it plays in cell division, an important event in carcinogenesis [28, 38, 46, 60, 78]. Particularities of TUBB3 expression and distribution are reported in various malignancies including small-cell and non-small cell lung cancer [49, 57, 69], ovarian [50], gastro-intestinal [4, 23, 85), pancreatic, kidney, prostate, breast [35, 36, 58, 78], head and neck [34] tumours.

In breast carcinoma, TUBB3 assessment indicates differential expression, according to the histological type, ER (oestrogen receptor) and PR (progesterone receptor) presence, and HER2/neu (human epidermal growth factor receptor 2) status [82]. TUBB3 supra-expression is related to poorly differentiated high-grade [82], triple-negative hormonal status, higher HER2 [36].

In clear-cell renal cell carcinoma [58], TUBB3 expression differs among the histological subtypes, being more frequent in papillary and chromophobe subtypes and oncocytoma compared to clear-cell subtype. In renal cell carcinoma (RCC) clear-cell, TUBB3 overexpression correlates to high Fuhrman grade, advanced stage, lymph node and hematogenous metastases, and shortened overall survival. On the contrary, in the papillary subtype, strong TUBB3

expression is associated to early tumour stage and overall survival. These variances can be explained by the differences in von Hippel-Lindan tumour suppressive (VHL) function involved in the carcinogenesis of clear-cell and papillary subtype, respectively, because VHL has a similar role to TUBB3, interfering in microtubule stabilization.

In the absence of molecular prognostic factors in bladder carcinoma, recent studies analyse different tubulin isotypes [7, 8, 45] following advanced stage patients' stratification, to customize the treatment. Another paper indicated the association between the overexpression of β-tubulin 1, β-tubulin 2, β-tubulin 3 and the tumour degree and stage, as well as shorter disease free survival [9].

TUBB3 analysis in a series of malignant melanoma showed high expression in 80% cases, but no correlation to classical clinical and pathological variables [73]. Surprisingly enough, TUBB3 decreased expression was associated to survival variables (overall survival and progression-free survival) [73], indicating an unfavourable prognosis.

Overexpression of TUBB3 in colorectal carcinoma, identified in tumour invasion margins [17, 22, 56], is correlated to the degree of tumour differentiation, lymphatic metastasis [85], poorer prognostic and lower overall survival [44].

Not at last, TUBB3 expression, 4.1 times higher in the uterine serous carcinomas compared to the ovarian serous ones, pleads for its prognostic value [15, 64].

From tubulin functions to cancer therapy

Due to its functions, tubulin became a target for the development of new therapeutic agents, used in adjuvant chemotherapy applied mainly in breast and ovarian cancer. Taxanes (Paclitaxel, Docetaxel, Cabazitaxel) are a class of drugs that stabilize the microtubules by interfering with spindle microtubule dynamics. Consequently, cell cycle is arrested, apoptosis initiated and tumour progression can be stopped [47, 48].

Recent research on the prognostic role of tubulin in several cancers supplements data that sustain its value for predicting the response to neo-adjuvant chemotherapy with taxanes.

Solid evidence published since the 2000s indicate that taxanes improve greatly the evolution of patients with various solid cancers, compared to other therapies. The most relevant example is the breast carcinoma [6, 53]. Favourable clinical results obtained by using this therapy (i.e. increased pathological complete remission rate in breast carcinoma) are conditioned by lower TUBB3 expression [6]. Additionally, *in vitro* and *in vivo* studies showed that TUBB3 can predict paclitaxel chemosensitivity in gastric cancer [83]. On the other hand, several reports showed that TUBB3 overexpression is directly involved in the resistance to taxanes. One of the first reports on this matter, published in 1997, demonstrates an altered expression of specific β-tubulin genes in taxol-resistant ovarian tumours [31, 65, 79]. Supplementary evidence for the relation TUBB3 - chemoresistance is supported by experimental studies, using cancer cell lines [3, 31, 39, 59] and clinical studies. The association between the TUBB3 overexpression, chemoresistance to taxanes and poor prognostic is documented in several malignancies, including breast [6, 20, 53, 76], gastric [80], pancreatic [37], colonic [44] and prostatic [55] carcinomas, clear-cell ovarian carcinoma [63], serous uterine carcinoma [64], uterine carcinosarcoma [5], thymic carcinoma [25, 51], non-small and small-cell lung carcinoma [13, 57, 66], locally advanced head and neck squamous-cell carcinoma [34], as well as in metastatic carcinomas of unknown primary site [15, 19, 43, 53, 71].

Nevertheless, discordant results also occur. Other papers show that strong TUBB3 expression is correlated to a favourable response to neo-adjuvant chemotherapy in non-small-cell lung carcinoma [70], clear-cell ovarian carcinoma [2, 15], ER negative breast carcinoma [82], HER2 positive breast carcinoma [24], advanced locally and metastatic breast cancer [16].

This different tumour behaviour reflects various biological characteristics of tubulin, including changes in the β-tubulin isotype composition in tumour sub-clones which influences tumour dynamics and the responsiveness of the chemical compounds that interfere with microtubules [30], in relation to the tumour stage (12). The variability of these results is deeply discussed by Jung and co-workers [24]. Their explanations take into account the important differences occurring in the design of various studies, namely the divergences in the group size, tumour stage, histological type and eligible patients [24]. Moreover, the immunohistochemical assessment of TUBB3 uses variable scores and cut-offs, being sometimes doubled by qRT-PCR [12].

Tubulin as potential prognostic marker and target therapeutic agent in thyroid tumours

TUBB3 study in thyroid tumours is limited to anaplastic-cell thyroid carcinoma (the most aggressive and rare solid thyroid tumour, with lethal evolution) in strict relation with the taxane therapy [1, 18]. A recent research [18] carried out under the coordination of the Working Group of Thyroid Cancer of the Spanish Society of Endocrinology and Nutrition and GETHI of the Spanish Society of Oncology established that for the operable anaplastic-cell thyroid carcinoma, adjuvant chemotherapy must include taxanes [74, 75], associated to doxorubicin, and cisplatin.

Published data referring to TUBB3 expression in PTC are practically inexistent. There are only two reports on TUBB3 immunohistochemical assessment [10, 11] in PTC, follicular adenoma, nodular hyperplasia and normal thyroid tissue. The authors

demonstrated the absence of TUBB3 in the normal follicular epithelium, nodular goitre and follicular adenoma, and a heterogeneous expression in PTC. They noted a negative TUBB3 reaction in the conventional, follicular or encapsulated variant of PTC, and a strong cytoplasmic TUBB3 expression in PTC widely infiltrative associated with fibrous stroma, particularly at the tumour invasive front, and in PTC moderately differentiated, with loss of cell polarity/cohesivity [10, 11]. Thus, the TUBB3 overexpression in PTC with "aggressive" histological features supports its potential role in invasion and metastasis.

Our study demonstrates low and moderate TUBB3 expression in 21 cases and high expression in 49 cases, and also the correlation with the histological risk subgroups, and tumour relapse. This fact suggests a relationship between TUBB3 and those histological characteristics of PTC that can explain the invasion and metastasis in the cases with poor prognosis. Starting from this idea, the taxanes' efficacy should be proved in those PTC cases that come out from the classic pattern of favourable prognostic, and need a personalized therapy.

Conclusions

TUBB3 overexpression could be a marker for un-favourable outcome in histological high-risk subgroup of PTC. In addition, the use of taxanes, as specific agents that ensure the microtubules stability, could be considered.

Acknowledgement

This work was funded by "Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania, under grant number 31584/2015.

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