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Expression of class III β-tubulin in malignant epithelial tumours: an immunohistochemical study using TU-20 and TuJ-1 antibodies

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Abstract: Class III β-tubulin has been discovered as a marker of early phases of neuronal differentiation in developmental conditions, as well as in different tumours of neuronal origin. More recently, the expression of class III β-tubulin molecule has been described as a marker of different types of malignant epithelial tumours. This study attempts to compare the immunostaining features of two different mouse monoclonal antibodies TU-20 and TuJ-1, both detecting class III β-tubulin, in a group of twenty bioptically evaluated carcinomas of various sites. The proposal that class III β-tubulin expression can correlate with the degree of tumour differentiation and thus could be potentially used as predictive marker of prognosis has been previously done; one of aims of our study was to confirm this hypothesis. Our results showed that both TuJ-1 and TU-20 antibodies displayed similar immunostaining profile and pattern within individual tumours. Surprisingly, we discovered that only 50% of tumours included in our group showed expression of class III β-tubulin, however, positive immunoreaction did not correspond with the degree of differentiation of individual tumours. In our group of carcinomas, the class III β-tubulin positivity was not related to the tumour site, histologic type of tumour or its grade.

Key words: Carcinomas - Class III β-tubulin - TU-20 - TuJ-1

Introduction

Class III β-tubulin, the molecule that belongs to tubulin superfamily [23], is a 50 kDa protein [1], encoded by gene located at the long arm of chromosome 16 [15]. Together with other tubulin superfamily members [25] it participates in formation of microtubular structures of the cytoskeleton. Class III β-tubulin expression recently represents an useful tool to investigate early phases of neuronal differentiation in human embryonic development [4,12,17] as well as in neoplastic tissues [16]. Wide spectra of different human neuronal and non-neuronal tumours exhibit class III β-tubulin expression in the neoplastic cells [15]. In some malignant tumours, the expression of class III β-tubulin correlates with the resistance to microtubule-acting chemotherapeutics [3,7,18,21]. While in neuronal tumours class III β-tubulin shows association with neuritogenesis and low growth potential, some authors associated class III β-tubulin expression in non-neuronal tumours with histologic signs of higher malignancy [15]. They hypothesised a relation between class III β-tubulin expression in tumour cells and lower degree of differentiation ("dedifferentiation") in some non-neuronal tumours. In that study, the presence of class III β-tubulin was detected immunohistochemically using TuJ-1 monoclonal antibody.

In order to confirm this hypothesis, we decided to study the expression of class III β-tubulin in a group of malignant epithelial tumours using another commercially available monoclonal antibody, TU-20, in addition to TuJ-1 immunostaining. Both antibodies have been raised against class III β-tubulin molecule [1], however, they recognize different epitopes. Comparative analysis of differences in staining properties of TU-20 and TuJ-1 in malignant epithelial tumours was also the objective of our work.

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Materials and methods

Patients. Twenty primary epithelial malignant tumours were enrolled in the recent study. The diagnosis of carcinoma was performed by histopathological analysis of the bioptic material. Selected paraffin-embedded tissue samples were obtained from the files of the Department of Pathology, 3rd Faculty of Medicine,

Table 1. Results of immunohistochemical detection of class III ß-tubulin with TU-20 and TuJ-1antibodies in goup of malignant epithelial tumours

No.	Tumour site	Histology	Gender	Age	Grade	TuJ-1	TU-20
1.	uterine cervix	squamous cell carcinoma	f	67	G2	2	2
2.		squamous cell carcinoma	f	65	G3	0	0
3.	uterine corpus	endometroid carcinoma	f	78	G3	1	1
4.		endometroid carcinoma	f	67	G1-2	0	0
5.	breast	invasive lobular and in situ ductal carcinoma (1)	f	79	G3	1	1
6.		invasive lobular carcinoma	f	83	*	0	1
7.	ovary	cystic serous papillary carcinoma	f	84	*	0	0
8.		cystic serous papillary carcinoma	f	68	*	1	2
9.		cystic serous papillary carcinoma	f	56	G3	1	1
10.	stomach	signet ring cell carcinoma	f	64	G3	0	0
11.	colon	tubular adenocarcinoma	f	72	G2	1	2
12.		tubular adenocarcinoma	f	44	G2	0	0
13.		tubular adenocarcinoma	m	77	G2	2	2
14.		tubular adenocarcinoma	m	53	G3	1	1
15.	anal canal	squamous cell carcinoma	f	62	G2-3	0	0
16.	pancreas	ductal adenocarcinoma	m	77	G2-3	0	0
17.	oesophagus	squamous cell carcinoma	m	58	G3	0	0
18.	bronchus	anaplastic carcinoma	m	54	G3	0	0
19.	head and neck	squamous cell carcinoma	m	57	G2	0	0
20.	prostate	prostatic adenocarcinoma, Gleason 9 (4+5)	m	79	G3-4	0	1

Charles University and Faculty Hospital King's Vineyard, Prague collected in years 1996-2004. The group of patients consisted of 13 women and 7 men whose age at the time of death ranged from 44 to 84 years (mean, 67 years). Examined tissue samples were obtained by surgical operation in 14 cases and by diagnostic biopsy in 6 cases. The results of histological examination are summarized in Table 1. The site of primary tumour was in five cases in colon and in anal canal, in three cases in ovaries, in two cases in female breast, endometrium and uterine cervix, and in one case each in prostate, pancreas, bronchus, stomach, oesophagus and head and neck region. Microscopically, the tumours were classified as adenocarcinomas and squamous cell carcinomas. One half of these tumours were scored as grade 3. All tumours showed local spread and metastatic dissemination. The autopsy was performed in all patients and histological investigation of tumour tissue obtained during the postmortal examination was consequently performed to compare original bioptic findings and final morphology of the tumour. In 19 patients (95%), generalisation of cancer with distant tumour metastases was evident, one patient with primary squamous cell carcinoma of oesophagus showed only regional lymph node metastases. Two cases of tumour duplicity were discovered in our patients. The patient with generalised prostatic adenocarcinoma was previously operated on for an adenocarcinoma of the caecum. No signs of this caecal tumour dissemination were observed during the autopsy. The patient with primary adenocarcinoma of the sigmoid suffered also from non-metastatic squamous cell cancer of penis. In one case of patient with primary lung carcinoma, bioptic specimen from enlarged lymph node with tumour metastasis was selected for immunohistochemical investigation, while primary tumour was discovered during autopsy in bronchial tree in the inferior lobe of the right lung. Histological investigation of autoptic material showed in all 20 cases identical cancer as that observed in the original biopsy. Data describing the undergone anti-tumour therapy had been collected from patients' hospital documentation.

Histology. All bioptic specimens were fixed in buffered formalin and embedded in paraffin. Five-micrometer thin sections were stained with haematoxylin and eosin. For immunohistochemical purposes the sections were placed on poly-D-lysine-coated glass slides.

Antibodies. Two different widely used primary mouse monoclonal antibodies recognizing class III β-tubulin, clones TU-20 and TuJ-1 were kindly gifted by Exbio, Prague, Czech Republic. TU-20 was diluted 1:100; the dilution of TuJ-1 was 1:50.

Immunohistochemistry. Standard immunohistochemical procedure was applied to all bioptic specimens. Different antigen retrieval procedures were applied to human cerebellar tissue,

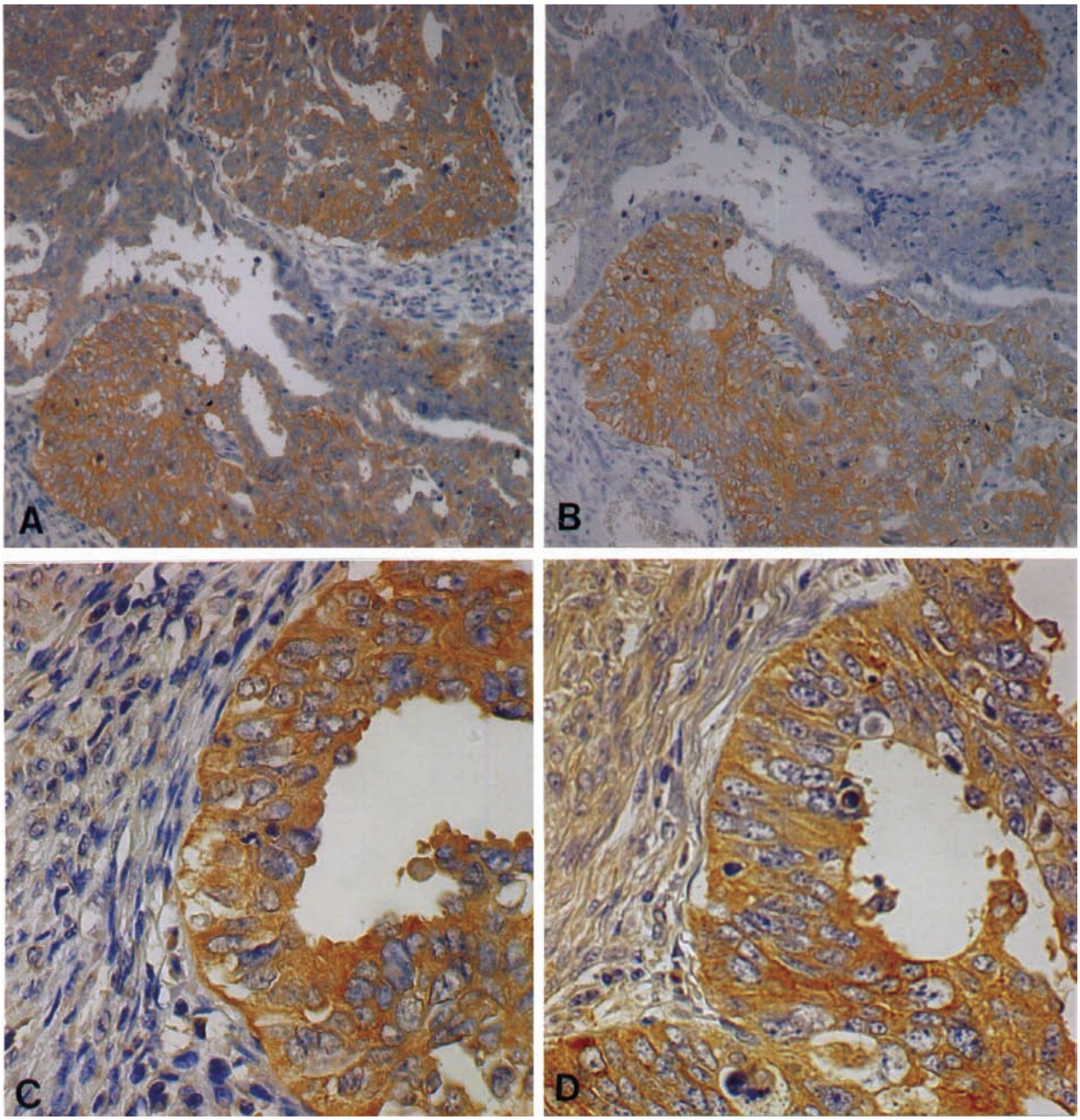

Fig. 1. Immunohistochemical detection of class III β-tubulin in human endometroid carcinoma (G3). Cytoplasmic staining of TU-20 (**A, C**) in tumour nests, compared with similar pattern of TuJ-1 immunostaining (**B, D**) in corresponding tumour parts in adjacent sections; original magnification × 200 (A and B) and × 600 (C and D).

which was used as positive control. Pre-treatment with 0.1 % trypsin solution in distilled water for 30 minutes at 37°C was chosen as optimal for TuJ-1, and boiling in a microwave oven in the citrate buffer, pH=6.0, for 3 × 3 minutes was applied as optimal antigen retrieval procedure for TU-20 antibody; other pre-treatment methods revealed varying degree of non-specific background and were abandoned. Both primary antibodies were applied to slides for 12 hours at 4°C. The En-Vision™ Kit purchased from DakoCytomation (Glostrup, Denmark) and 3,3-diaminobenzidine (DAB, Fluka, Buchs, Switzerland) were used to visualize immunohistochemical reactions. The slides were counterstained with Harris haematoxylin. Sections incubated without primary antibodies were used as negative controls. Immunostaining results were evaluated semiquantitatively in the whole tissue sections as follows: 0 - no positive staining; 1 - less than 50% cells positive; 2 - more than 50% cells positive.

Results

Table 1 summarizes the cases, location, microscopic appearance as well as results of immunostaining for both TU-20 and TuJ-1. Ten cases (50%) were negative for class III β-tubulin after application of both antibodies, TU-20 and TuJ-1. In 6 of these negative cases, the tumours were scored as high-grade G 2-3 and 3; four tumours displayed the grade G1 or G2. Positive immunostaining with both antibodies was observed in 8 cases (40%); four of them were scored as G3 tumours (Fig. 1A-B). In a case of high-grade prostatic adenocarcinoma and one invasive lobular breast carci-

noma, positive immunostaining was detected in scattered neoplastic cells with TU-20 only, while immunostaining of class III β-tubulin with TuJ-1 antibody was negative. In all positive cases, a diffuse fine immunostaining was observed in cytoplasm of neoplastic cells. In one positive case of squamous cell carcinoma of uterine cervix (no. 1) almost all neoplastic cells revealed positive cytoplasmic immunoreactivity reaction with moderately varying intensity with both antibodies. In case no. 13, where endoscopic biopsy obtained from colonic adenocarcinoma was investigated, a weak cytoplasmic staining with both TU-20 and TuJ-1 was detected in almost all neoplastic cells. Remaining immunopositive tumours, which were classified as adenocarcinomas, revealed varying intensity of immunoreaction within the tumour mass; patchy or focally pronounced cytoplasmic immunoreaction for class III β-tubulin was observed in individual neoplastic cells and/or in small clusters of neoplastic cells in six cases. The intensively immunoreactive areas were dispersed within the tumour mass while in the remaining neoplastic cell population the immunoreaction showed either low intensity of staining or neoplastic cells were negative. Comparing the immunostaining features of TU-20 and TuJ-1, both monoclonal antibodies showed almost identical pattern and distribution of immunoreactivity within the tumour mass; in two tumours (no. 8 and 11) slightly increased number of neoplastic cells reacting with TU-20 was observed compared with TuJ-1.

The data on oncological treatment were available from 18 patients, three of them received chemotherapy in combination with other forms of treatment, while in 15 patients surgical therapy, actinotherapy or symptomatic palliative treatment was applied without administration of chemotherapy. In one of these three patients (no. 11), no detailed information about type of used chemotherapy was available, in two other patients (no. 8 and 9), both suffering from ovarian primary cancer, administration of Taxol was mentioned in clinical documentation. One patient (no. 8) underwent this therapy with common dosage, while in patient no. 9, the therapy had to be abandoned due to its intolerance by patient. Both cases no. 8 and 9 revealed the presence of class III β-tubulin in neoplastic cells using both TU-20 and TuJ-1.

Discussion

Class III β-tubulin has been proposed as a useful marker of early phases of neuronal differentiation in developmental studies and in tumours of neuronal origin. Immunoreactivity for class III β-tubulin in embryonic type neuronal/neuroblastic tumours like medulloblastoma is associated with more pronounced neuronal differentiation and decreased cell proliferation rate [11], while in the group of gliomas, class III β-tubulin expression correlates with higher grade of histological malignancy and correspondingly with increased proliferation of tumour cells [14].

The expression of class III β-tubulin has also been detected in different types of epithelial tumours. Relatively high percentage of neuroendocrine tumours (carcinoids) of the respiratory and gastrointestinal tract reveal positive immunostaining for class III β-tubulin [10,13]. This positivity reflects neuronal differentiation of these malignant epithelial cells. According to previously well-known positivity of neuron-specific enolase and synaptophysin [5], widely used as markers of neuronal differentiation in carcinoid tumours, positive immunoreactivity for class III β-tubulin represents a new histopathological diagnostic tool. Recently, expression of class III β-tubulin has been extensively studied in other malignant epithelial, non-neuroendocrine, tumours. The role of class III β-tubulin overexpression in the process of tumour resistance to taxol-based chemotherapeutic drugs, such as paclitaxel and docetaxel has been studied *in vitro* [2,7,8,22,24] as well as in clinical conditions [3,6,9,19,20,21].

Increased expression of class III β-tubulin in malignant epithelial tumours of higher grade was hypothesised earlier [15]. Detection of class III β-tubulin in human malignant epithelial tumours has been proposed as a sign of higher malignant potential and poorer prognosis. This concept was based on immunohistochemical detection performed mainly with TuJ-1 mouse monoclonal antibody, raised against the last 14 aminoacids at the C-terminal part of chicken class III β-tubulin. Another commercially available monoclonal antibody against class III β-tubulin, TU-20, recognizes the last eight aminoacids (ESESQGPK), corresponding to C-terminal part of the human class III β-tubulin. Differences in immunoreactivity of TuJ-1 and TU-20, respectively, have been previously studied and described [1]. The authors suggested different reasons for this phenomenon, namely specific masking of antigenic determinant by posttranslational modifications and topical binding of microtubule-associated proteins. On the contrary, the results of our study show that both antibodies reveal almost identical intensity and pattern of immunoreactivity in neoplastic epithelial cells. In our opinion, these findings exclude either potential crossreactivity, or false positivity, which could appear in cases when only one antibody was used under the experimental conditions.

The results of our study showed that only approximately one half of randomly selected group of malignant epithelial neoplasms revealed positive immunostaining for class III β-tubulin. Immunostaining varied in intensity even within the tumours of identical organ and histogenetic origin. Both positive and negative tumours were observed in one topographical subgroup

of epithelial neoplasms, for example intestinal and breast carcinomas. The results of our study suggest that positive immunostaining for class III β-tubulin does not exactly correspond to any primary cancer site and grade. We did not confirm previously proposed hypothesis of increased expression of class III β-tubulin in less differentiated carcinomas. We observed no correlation between class III β-tubulin expression and the degree of differentiation (grade) of individual tumours included in our group. Our data indicate that the occurrence of class III β-tubulin in malignant epithelial tumours seems to be random; however, detailed studies with larger number of included cases have to confirm that statement. The usefulness of class III β-tubulin immunoreactivity in histopathological differential diagnostics of adenocarcinomas from different primary sites should also be further studied in larger tumour groups.

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