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PTTG and Ki-67 expression in pituitary adenomas

Ekspresja PTTG i Ki-67 w gruczolakach przysadki

Introduction: The unpredictable biology of pituitary adenomas makes it a therapeutic challenge. Moreover, histopathology of pituitary carcinomas and locally invasive adenomas are indistinguishable from benign tumors and a new marker which would enable to differentiate those lesions is vital. The aim of the study was to evaluate Ki-67 and PTTG (pituitary tumour-transforming gene) expression in pituitary adenomas and their application as markers of tumour aggressiveness.

Material and methods: A retrospective analysis of 55 patients: 32 females (58%) and 23 males (42%), mean age 50±16 years who underwent pituitary tumor surgery between 2003-2012. Ki-67 and PTTG indices were determined by immunohistochemical staining. Magnetic resonance imaging or computed tomography was performed beforehand and one year after surgery to figure a potential tumour progression, tumour size and correlation to adjacent tissues.

Results: The expression of Ki-67 and PTTG was revealed in cell nuclei in 88% and 85% of adenomas, respectively. The median Ki-67 and PTTG indices were 1.4 and 1.0, respectively ($p=0.006$). In the group with macroadenoma as compared with the group with microadenoma, median Ki-67 index was higher (1.4% vs. 1.03%; $p=0.02$). We did not find correlation between both Ki-67 and PTTG indices and tumour progression. Tumours with positive immunostaining towards FSH revealed lower Ki-67 and PTTG indices than the rest with a negative one (0.6% vs. 1.84%, $p=0.0004$ and 0.67% vs. 1.23%, $p=0.047$; respectively). However, PTTG index was higher in the group with acromegaly as compared to the group with clinically non-functioning pituitary adenoma (NFA) (1.28% vs. 0.35%; $p=0.02$).

Conclusions: Positive nuclear expression of Ki-67 and PTTG was observed in the majority of pituitary adenomas. Only higher Ki-67 expression was related to the tumour invasiveness found on MRI/CT. Tumour progression was not related to both Ki-67 and PTTG expression.

Wstęp: W praktyce klinicznej zarówno przewidywanie zachowania gruczolaków przysadki jak ich leczenie jest dużym wyzwaniem. Co więcej, badanie histopatologiczne nie pozwala odróżnić raka przysadki, miejscowo naciekającego gruczolaka od zmiany łagodnej. Celem pracy była ocena ekspresji Ki-67 oraz PTTG (pituitary tumour transforming gene) w guzach przysadki i ich znaczenia jako markerów agresywności tych gruczolaków.

Materiał i metody: Badanie miało charakter retrospektywny. Objęło 55 badanych pacjentów: 32 kobiety (58%) i 23 mężczyzn (42%), średnia wieku 50±16 lat; po operacji neurochirurgicznej z powodu gruczolaka przysadki pomiędzy 2003 a 2012 rokiem. W oparciu o badanie immunohistochemiczne preparatów obliczono indeksy Ki-67 i PTTG. Celem oceny wielkości guza, wzajemnej zależności z sąsiadującymi tkankami oraz ewentualnej wznowy, wykonano rezonans magnetyczny lub tomografię komputerową przysadki przed i 1 rok po zabiegu.

Wyniki: Wykazano jądrową ekspresję w komórkach gruczolaka przysadki w przypadku Ki-67 u 88% i PTTG u 85%. Mediany indeksu Ki-67 i PTTG wynosiły odpowiednio 1,4% i 1,0% ($p=0,006$). W grupie z makrogruczolakiem, indeks Ki-67 był wyższy w porównaniu do grupy z mikrogruczolakiem (1,4% vs 1,03%; $p=0,02$). Nie stwierdzono zależności pomiędzy indeksem Ki-67, PTTG a progresją guza. W przypadku guzów z dodatnim immunohistochemicznym odczynem w kierunku FSH, indeksy Ki-67 i PTTG były niższe (0,6% vs 1,84%; $p=0,0004$ oraz 0,67% vs 1,23%; $p=0,047$). Indeks PTTG był wyższy wśród guzów z pozytywnym odczynem immunohistochemicznym w kierunku substancji hormonalnych (1,28% vs. 0,35%; $p=0,02$).

Wnioski: Jądrowa ekspresja Ki-67 i PTTG występowała u większości badanych i wykazywała wzajemną pozytywną korelację. Jedynie większa ekspresja Ki-67 wykazywała znamienny związek z wielkością i cechami inwazyjności guza w badaniu MRI/CT. Nie wykazano związku obu indeksów z progresją guza.

Introduction

Pituitary tumours, in majority of cases benign adenomas, account for 10-25% of intracranial tumours, their prevalence in the general population being 0.02% [1,2]. Anatomically, these tumours are classified as microadenomas (diameter <1cm) or macroadenomas (diameter ≥1cm) [1,3].

Clinically, pituitary tumours may be accompanied by hormonal activity or symptoms of "mass effect", such as invasion of the surrounding tissues (e.g. supra-sellar extension, sinus cavernous invasion and optic nerve compression), causing visual disturbances or headache. Compression of non-tumour pituitary results in varying degrees of hypopituitarism [2]. Based on physical examination and blood hormone levels, pituitary tumours can be broadly classified as either functioning (FPA) or non-functioning pituitary adenomas (NFPA) - comprising approximately 30% of all adenomas [1]. In about 10% of autopsies small pituitary adenomas are found, most of them having had no clinical manifestation [3].

The predicted hormonal activity of the tumour is not always confirmed by postoperative immunohistological examination. Within WHO classification (2004) pituitary adenomas are divided according to their hormone secretion as follows: *Pit-1 family* (prolactinoma, PRL secretion - 50%; somatotropinoma, GH secretion - 15-20%; thyrotropinoma, TSH secretion - 1%; plurihormonal adenoma), *ACTH family* (corticotropinoma - 5-10%), *gonadotropin family* (gonadotropinoma, LH, FSH secretion - 1%), or *unclassified adenomas* (hormone negative/null cell adenoma, NCA or unusual plurihormonal adenoma) [2,4,5,6].

According to WHO, pituitary tumours are classified according to their morphology features as typical or atypical adenomas (the latter being characterised by invasive growth, increased mitotic index, Ki67 labelling index >3% and significant positive p53 nuclear staining) and carcinomas [4,7,8]. Pituitary carcinomas are very rare (0.1-0.2% of all cases of pituitary tumors). They are diagnosed if cerebrospinal or systemic metastases are observed [1,4,8]. Most pituitary carcinomas develop from invasive recurrent prolactinomas and corticotropinomas, with very poor prognosis [3]. The therapy of aggressive pituitary tumours and carcinomas is challenging. Besides surgery (sometimes aided by intraoperative low-field MRI), medical treatment, radiotherapy and new cytostatic temozolomide, are used [9,10].

The clinical outcome of pituitary adenomas does not always correlate with the histology of those tumours, some being invasive (30-45%) and some being clinically aggressive [8]. In invasive adenomas cellular pleomorphism, nuclear atypia, increased cellularity, focal necrosis and mitotic figures are not always present [8]. There are no defined histological criteria to distinguish between benign and malignant tumours, especially between atypical adenomas and pituitary adenocarcinomas.

The inadequacy of WHO's classification of pituitary adenomas and the need to re-examine it is currently under debate

[4,5,8,11,12]. Trouillas et al. [8] propose a classification of adenomas into non-invasive, non-invasive and proliferative, invasive and non-proliferative and metastatic, while Pasquel et al. [13] suggest that atypical adenomas should be named carcinoma *in situ* or pre-metastatic cancer.

Typically the term "aggressive" pituitary adenoma is used in clinical practice in the case of a tumour of large size with local mass effect, local invasion, post-operative recurrence, sustained hormonal function despite excision, or resistance to treatment - those being potential indicators of aggressiveness of atypical adenomas [3,7].

The search for a standardized and optimal marker which would consistently correlate with biological tumour behaviour, to enable proper diagnosis, more effective treatment and proper rational post-operative observation, is still under way. Such potential indicators of tumour behaviour are markers of proliferation and angiogenesis. Apart from documented Ki-67 (MIB-1 antibody), topoisomerase II alpha, COX-2, and Bcl-2 were studied [1,6] however, as yet without consistent correlation with tumour invasiveness or recurrence found on MRI or CT [5]. Pawlikowski et al. found prevalent expression of FSHR in invasive and proliferating adenomas as compared with non-invasive and non-proliferating tumours [14]. Pituitary tumour transforming gene (PTTG), vascular endothelial growth factor (VEGF), prothymosin alpha and AIP are new promising markers of biological tumour behaviour.

Ki-67 is a nuclear antigen expressed in G1, S, G2 and M phases of the cell cycle and recognized by the commercially available MIB-1 antibody [15,16]. The Ki-67 labelling index can vary between <1% and 23% [17]. PTTG is an oncogenic protein of the securin family, located on chromosome 5q33. The function of PTTG is not well known. According to some authors, it can induce fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) and inhibits premature sister chromatid separation which is crucial in normal cell mitosis [2,17]. Over-expression of PTTG increases cell proliferation and promotes tumour formation in various tissues, such as the thyroid, ovary, breast, prostate, lung, oesophagus, colon and central nervous system [2,17]. In pituitary tumours, PTTG appears to be a molecular marker of invasiveness and angiogenesis [17]. The PTTG family is comprised of PTTG1 (the most studied), PTTG2, and PTTG3. In immunocytochemistry PTTG is detected in the nucleus (predominantly) and in the cytoplasm [17].

The aim of this study was:

1. To determine the usefulness of PTTG as a prognostic factor of pituitary adenoma progression;
2. To evaluate the relationship between the proliferative activity of pituitary adenomas, as quantified by Ki-67 and PTTG indices, and their aggressive biology and clinical data; and,
3. To investigate the correlation between the PTTG labelling index and tumour size and local invasiveness.

Material and methods

A retrospective analysis was undertaken of clinical records, histopathological and immunohistochemical slides of pituitary adenomas in 55 patients of the Department of Endocrinology, University Hospital in Kraków, who underwent transsphenoidal resection of pituitary adenoma and/or craniotomy in the Department of Neurosurgery, University Hospital in Kraków, between 2003-2012. The diagnosis was established by clinical, biochemical and radiological observations and confirmed by surgery and histopathology. A retrospective analysis of the studied group is presented in table I.

All patients underwent magnetic resonance imaging (MRI) or computed tomography (CT) prior to and one year after surgery, to determine tumour recurrence. Tumour size, defined as its largest dimension, and invasion of neighbouring tissues (destruction of sella turcica, penetration of cavernous sinus, optic chiasm compression, suprasellar propagation), were studied by MR or CT imaging. Tumour invasiveness was defined by radiological criteria [18,19] and from the surgeon's description in the medical records. Tumour progression was evidenced by its recurrence or by increase of the remnant tumour size in MRI/CT.

The surgical specimens were routinely processed, fixed in neutral buffered formalin, embedded in paraffin and placed on slides. In hematoxyline-eosine staining, 18 (33%) of adenomas were acidophilic while 34 (62%) were chromophobic. For immunocytochemistry, sections were incubated, *inter alia*, with primary mouse monoclonal antibody Ki-67 clone MIB-1 antibody (Dako, M7240, 1:25) and with the primary monoclonal Securin antibody (PTTG, Novocastra, 1:25). To confirm the specificity of the primary antibody, positive and negative control tests were performed, following the manufacturer's instructions.

Ki-67 and PTTG-stained sections were evaluated manually using an optical microscope (Nikon Optishot-2). Ten fields were selected in regions with the highest concentration of positive nuclei and examined at magnification 400x. Each field covered 700 to 3800 cells, depending on the cellularity of the tumour specimen. Ki-67 and PTTG indices were defined as the respective average percentages of positively stained nuclei. Less intensive PTTG immunostaining was also scored in adenoma cell cytoplasm (c-PTTG). In over 90% of samples with positive nuclear PTTG immunoreactivity, positive c-PTTG immunoreactivity was also observed (Fig. 1).

Statistical analysis (analysis of variance, student's t-test, Pearson's r correlation, Wilcoxon test, Spearman's rank correlation) was performed using the R statistical package (a free software environment for statistical computing and graphics).

This study has been approved by the Local Bioethics Committee of the Jagiellonian University Medical College (certificate KBET/169/B/2010).

Results

The expression of PTTG and Ki-67 in pituitary adenoma patients, grouped accor-

Figure 1
Picture 1. 400x magnification. The strong nuclear reaction to PTTG in 2 cells and mixed (nuclear+cytoplasmatic) in 1 cell. Picture 2. 400x magnification. The stronger nuclear (in 4 cells) and weaker cytoplasmatic reaction (in the rest of cells) to PTTG.

Obraz 1, powiększenie 400x. Silna reakcja jądrowego PTTG widoczna w dwóch komórkach oraz mieszana (jądrowa i cytoplazmatyczna) widoczna w jednej komórce.

Obraz 2, powiększenie 400x. Silniejsza reakcja jądrowego PTTG widoczna w czterech komórkach oraz słabsza reakcja cytoplazmatycznego PTTG widoczna w pozostałych komórkach.

Table I
Characteristic of the studied group.
Charakterystyka badanej grupy.

General data	Age, years	50±16 (21-80)
	Gender: Female/Male (%)	32 (58%)/ 23 (42%)
Imaging results	Largest dimension of tumor [mm]	100
	Minimal dimension of tumor [mm]	4
	Microadenoma (%) / Macroadenoma (%)	7 (13%)/48 (87%)
	Optic chiasm compression (%)	28 (51%)
	Local invasion of tumor	43 (78%)
Final clinical classification of pituitary adenomas in patient group	Clinically non-functioning adenoma (NFPA) (%)	20 (36%)
	prolactinoma (%)	2 (4%)
	acromegaly (%)	31 (56%)
	acromegaly + prolactinoma (%)	2 (4%)
	Cushing disease (%)	0
	ganadotropinoma (%)	0
	thyrotropinoma (%)	0
	silent-ACTH adenoma (%)	0
Immunohistochemical findings	Hormonal positivity	42 (78%)
	NCA	13 (22%)
	GH positive	27 (50%)
	PRL positive	18 (33%)
	ACTH positive	8 (15%)
	TSH positive	6 (11%)
	FSH positive	8 (15%)
	LH positive	11 (20%)
	Plurihormonality	13 (24%)
	Histopathological features of malignancy*	7 (13%)
Clinical observation after 1 y	Progression	16 (29%)

*Classic histopathological features of malignancy, like nuclear polymorphism, presence of mitotic figures, high cellularity, cytological atypia, presence of necrosis might be an indicator of more aggressive behavior.

*Klasyczne histopatologiczne cechy złośliwości, takie jak polimorfizm jądrowy, obecność figur mitotycznych, znaczna liczba komórek, cytologiczna atypia, obecność zmian nekrotycznych, mogą być wskaźnikami zwiększonej agresywności guza.

Table II
Ki-67 and PTTG expression in the studied group.
Ekspresja Ki-67 i PTTG w badanej grupie.

	Ki-67	PTTG	p - value
Frequency	88%	85%	
Median index [%]	1,4%	1,0%	p=0,006

ding to their final diagnosis (acromegaly, prolactinoma, NFPA) is shown in figure 2. The values of studied indices are shown in the table II.

Progression of the tumour within 12 months of the observation was found in 16 cases (29%). However, we did not find any significant difference between median Ki-67

index in tumours with and without progression (1.65% vs. 1.22%; p=0.896). Similarly there was no significant difference between median PTTG index in tumours with and without progression (0.97% vs.1.12%; p=0.82)

PTTG and Ki-67 expression were correlated ($\rho=0.37$; p=0.006), as illustrated in figure 3.

The group with macroadenoma consisted of 48 (87%) patients. In comparison to the group with microadenoma (n=7) Ki-67 expression was higher (1.4% vs. 1.03%; p=0.019) (Fig. 4).

In patients with macroadenoma, no correlation between Ki-67 or PTTG and local invasion, tumor size and progression was found.

The Ki-67 index was significantly higher in locally invasive tumors (1.39% vs. 0.64%; p=0.034). No relationship between optic chiasm destruction and Ki-67 or PTTG expression was found.

PTTG index was significantly higher in patients with positive immunohistochemical hormonal expression (1.2% vs. 0.1%; p=0.07) in comparison to NCA, while in case of Ki-67 there was no relationship. However, median Ki-67 and PTTG indices were higher in patients with negative expression towards FSH than with positive expression towards FSH (1.84% and 1.23% versus 0.6% and 0.67% respectively; p=0.0004 and p=0.047 respectively).

In a group of patients with positive expression of GH, local invasion was more often found than in the group with negative expression towards GH (p=0.002).

According to their clinical diagnosis we divided patients into two main groups: with acromegaly and NFPA (n=31, 56% and n=20, 36%) respectively). There was significant difference in median PTTG between those groups; acromegaly – 1.28% and NFPA – 0.35%; p=0.002 (Fig. 2,5).

All NFPA patients presented local invasion. There was a significant difference in local invasion frequency between those groups: with acromegaly - 19 (61%), with NFPA 20 (100%); p=0.004 (Tab. IV).

We found correlation between PTTG expression and age. PTTG index was considerably higher in younger patients (p=0.0019). Whereas, Ki-67 index was higher in women (p=0.039).

Cytoplasmic PTTG (c-PTTG) expression was not related to the invasiveness nor hormonal activity of examined tumours.

Discussion

Our study contributes to the on-going search for a new biological marker useful in early diagnosis of pituitary adenoma and carcinoma. So far, no marker to independently predict aggressive behaviour of such tumours has been found. Correlation between Ki-67 and tumours recurrence was observed in several studies [8,20,21]. The statistically significant correlation between Ki-67 index and tumour size and invasiveness (higher in macroadenomas) found in our study agrees with some of these observations [11,21]. However, in agreement with other studies [22,23,24], we were not able to correlate tumour progression with the Ki-67 index.

Figure 2
Expression of PTTG and Ki-67 in patients with pituitary adenoma grouped according to their final diagnosis.
Ekspresja PTTG oraz Ki-67 u pacjentów z gruczolakiem przysadki w zależności od ostatecznego rozpoznania.

Figure 3
Correlation between PTTG and Ki-67 indices.
Korelacja indeksów PTTG oraz Ki-67.

Table III
Histopathological features and radiological progression (after 1 year of observation) of patients with or without local invasion found on MRI/CT.

Obraz histopatologiczny oraz progresja w badaniach obrazowych (po 1 roku obserwacji) u pacjentów z obecną oraz nieobecną miejscową inwazyjnością guza w badaniu MRI/CT.

		No invasion (n=12, 22%)	Invasion (n=43, 78%)	p - value
Tumor progression on MRI/CT		2 (16,6%)	14 (32%)	p = 0,47
	Ki-67	0.64%	1.39%	p = 0,034
Histopathological markers of proliferation	PTTG	1.0%	1.12%	p = 0.75
Histopathological indicators of aggressiveness		1 (8.3%)	6 (14%)	p = 1

Table IV
Histopathological and MRI/CT findings (after 1 year of observation) of patients with acromegaly and NFPA (non-functioning pituitary adenomas).

Wyniki badania histopatologicznego oraz obrazowego (po 1 roku obserwacji) u pacjentów z akromegalia oraz NFPA.

		Acromegaly (n=31, 56%)	NFPA (n=20, 36%)	p-value
	Local invasion	19 (61%)	20 (100%)	0.001
MRI/CT findings	Tumor progression	9 (29%)	6 (30%)	1
Histopathological markers of proliferation	Ki-67	1.21	1.67	0.4
	PTTG	1.28	0.35	0.002
Histopathological indicators of aggressiveness		6 (19%)	1 (5%)	0.22

We note the limited number of studies concerning PTTG expression in pituitary tumours.

PTTG and pituitary adenoma progression

Filippella et al. [25] studied the expression of PTTG and Ki-67 in 45 pituitary adenomas reporting correlation between PTTG and Ki-67 and their both cut-off value which could predict recurrence was 2.9%.

A similar study, but concerning NFPA only, was reported by Noh et al [26], who investigated 35 pituitary adenomas finding correlation between Ki-67 and PTTG expression with tumor recurrence. We found a statistically significant relationship between PTTG and Ki-67 expression, but no relevant association among PTTG and tumour progression or tumour size, in agreement with other studies [22,23,24,27].

PTTG, local invasiveness and recurrence

It is generally accepted, and also supported by our study, that invasive adenomas have a higher proliferation rate and Ki-67 indices. In a study of correlation between PTTG and local invasion Sanches-Tejada et al. [22] using a sensitive real-time reverse transcriptase polymer chain reaction (RT-PCR) assay, detected PTTG in their samples, finding PTTG expression to be lower in invasive than in non-invasive tumours, however not being significantly correlated with tumour recurrence.

Wierinckx et al. [23], who examined PTTG in prolactinomas only, found no correlation between PTTG and clinical course (invasiveness and recurrence). While their findings are somewhat similar to ours, in that we were not able to find a correlation between PTTG index and tumour recurrence or invasiveness. Although, we noted a higher PTTG expression in acromegalic patients than in NFPA, it was the group with NFPA which had a higher rate of invasiveness.

PTTG and hormonal activity

Hunter et al. [28] using techniques based on RT-PCR, showed that levels of PTTG mRNA in somatotropinomas were significantly higher than those in NFPA [28]. Also Zhang et al. [29] evaluated the expression of PTTG with RT-PCR in 54 pituitary adenomas and in normal pituitary tissue, finding that PTTG over-expression correlated with tumour invasiveness in FPA (which had invaded the sphenoid bone). Although results of McCabe et al. [24] contradicted those of Zhang et al. [29] (higher PTTG mRNA in NFPA), our study supports a positive correlation between the PTTG and FPA and PTTG and somatotropinomas.

Ramirez and al. [27] analyzed NFPA in 74 patients (average Ki-67 index value of 1.49%), in whom significant dependence of this index on tumor size (higher indices at tumour size > 30mm) and tumour recurrence were observed. They did not find such correlation with respect to PTTG [27]. In our study, the group with NFPA consisted of 20 samples and the median Ki-67 index was 1.67%. However, among NFPA we did not prove any correlation in between Ki-67 or PTTG and tumour size >30mm.

Figure 4
Distribution of Ki-67 index in patients with microadenoma and macroadenoma.
Rozkład indeksu Ki-67 u pacjentów z mikrogruczolakiem i makrogruczolakiem.

Figure 5
Distribution of PPTG index in patients with acromegaly and NFPA.
Rozkład indeksu PPTG u pacjentów z akromegalią oraz NFPA.

PTTG and macroadenoma

Jia et al.[30] investigated the relationship between PTTG and invasiveness in 70 patients with macroadenomas and using RT-PCR demonstrated positive correlation between PTTG and local invasion. In our study of 48 patients with macroadenoma we did not find such a relationship.

We are aware of the relatively short follow up and the small and heterogeneous group of analysed tumours as the limitations of our study.

While PTTG and Ki-67 appear to play an important role in pituitary tumorigenesis, there is no consensus as to the distinction between benign and aggressive pituitary adenomas. The limitations of PTTG and Ki-67 as markers are probably connected with their multiple cellular functions. Prospective exploration of proliferative markers in recurrent and invasive pituitary adenomas could lead to effective targeted therapy of these tumours.

Conclusions

1. Expression of Ki-67 and PTTG was observed in the majority (88% and 85%

respectively) of pituitary adenomas.

2. Ki-67 and PTTG indices were correlated in the studied group.

3. Neither Ki-67 nor PTTG expression, were related to tumour progression.

4. Only Ki-67 index was higher in macroadenomas and in locally invasive adenomas as compared to microadenomas and non-invasive adenomas, respectively.

5. PTTG index was significantly higher in patients with positive immunohistochemical hormonal expression, as compared to NCA.

6. PTTG index was significantly higher in acromegalic patients, as compared with patients diagnosed as having NFPA.

7. All patients with NFPA had locally invasive pituitary adenoma.

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