



Markers of proliferation and invasiveness in somatotropinomas

Markery proliferacji i inwazyjności w guzach przysadki wydzielających hormon wzrostu

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Abstract

Introduction: In the search for markers of invasiveness of pituitary adenomas, we studied the expression of Ki-67 antigen, TOPO 2A (topoisomerase 2 alpha), AIP (Aryl Hydrocarbon Receptor-Interacting Protein), and VEGF (Vascular Endothelial Growth Factor) in somatotropinomas.

Material and methods: We retrospectively studied a group of 31 patients who underwent pituitary tumour surgery. Expression of Ki-67, TOPO 2A, AIP, and VEGF in surgical specimens was determined by immunohistochemistry. Relations between quantitatively determined markers and clinical symptoms, tumour features, and MR imaging, were analysed. Acromegaly was confirmed by hormonal tests in all patients studied. Local invasiveness (cavernous sinus penetration, optic chiasm compression or suprasellar extension) was observed in 18/31 patients (58.1%).

Results: Ki-67 was expressed in 77.4%, TOPO 2A in 87.1%, AIP in 83.8%, and VEGF in 87.1% of 31 cases of somatotropinoma. Median values of Ki-67, TOPO 2A, AIP, and cytoplasmic VEGF indices, were 1.2% [IQR = 2.2], 1.5% [IQR = 1.6], 21.26% [IQR = 20.1], and 20.4% [IQR = 15.4], respectively.

Ki-67, TOPO 2A, AIP, and VEGF expression was not correlated with age nor with patient gender ($p > 0.05$). Only Ki-67 and TOPO 2A correlated with tumour size (for Ki-67: $r = 0.42$, $p = 0.025$; for TOPO 2A: $r = 0.53$, $p = 0.003$). Ki-67 and TOPO 2A levels were significantly higher in invasive compared to noninvasive somatotropinomas (Ki-67 mean values: $1.85 \pm 1.33\%$ vs. $0.95 \pm 1.07\%$, $p = 0.024$; TOPO 2A mean values: $2.19 \pm 1.63\%$ vs. $1.45 \pm 1.23\%$, $p = 0.011$).

Conclusions: Ki-67, TOPO 2A, AIP, and VEGF were expressed in over 70% of all somatotropinomas. Only Ki-67 and TOPO 2A expression correlated with tumour size and tumour invasiveness. (*Endokrynol Pol 2018; 69 (2): 182–189*)

Key words: pituitary adenoma, markers of proliferation, Ki-67, TOPO 2A, AIP, VEGF

Streszczenie

Wstęp: W poszukiwaniu markerów inwazyjności gruczołów przysadki badano ekspresję antygenu Ki-67, TOPO 2A (topoisomerase 2 alpha), AIP (Aryl Hydrocarbon Receptor-Interacting Protein) oraz VEGF (Vascular Endothelial Growth Factor) w guzach wydzielających hormon wzrostu.

Materiał i metody: Badano retrospektwnie grupę 31 pacjentów, operowanych z powodu guza przysadki. Ekspresje Ki-67, TOPO 2A, AIP oraz VEGF badano immunohistochemicznie w tkankach uzyskanych chirurgicznie. Analizowano relacje między ilościową ekspresją markerów a objawami klinicznymi, cechami guza i obrazem MR. Testami hormonalnymi potwierdzono akromegalię u wszystkich badanych pacjentów. Miejscową inwazyjność, zdefiniowaną jako naciekanie zatoki jamistej, kompresję skrzyżowania wzrokowego, wzrost nadsiatkowy guza obserwowano u 18/31 (58,15) pacjentów.

Wyniki: Ekspresję Ki-67 stwierdzono w 77,4%, TOPO 2A w 87,1%, AIP w 83,8%, oraz VEGF w 87,1% przypadków samatotropinoma (n = 31). Wartości median indeksów Ki-67, TOPO 2A, AIP i cytoplazmatycznego VEGF wynosiły 1,2% [IQR = 2,2], 1,5% [IQR = 1,6], 21,26% [IQR = 20,1] i 20,4% [IQR = 15,4], odpowiednio. Indeksy Ki-67, TOPO 2A, AIP i VEGF nie korelowały z wiekiem i płcią pacjentów ($p > 0.05$). Tylko Ki-67 i TOPO 2A korelowało z wielkością guza (dla Ki-67: $r = 0,42$, $p = 0,025$; dla TOPO 2A: $r = 0,53$, $p = 0,003$). Indeksy Ki-67 oraz TOPO 2A były znacznie wyższe w guzach inwazyjnych w porównaniu z guzami nieinwazyjnymi (średnie Ki-67: $1,85 \pm 1,33\%$ vs $0,95 \pm 1,07\%$, $p = 0,024$ oraz średnie TOPO 2A: $2,19 \pm 1,63\%$ vs $1,45 \pm 1,23\%$, $p = 0,011$).

Wnioski: Ekspresję Ki-67, TOPO 2A, AIP i VEGF stwierdzono w ponad 70% wszystkich somatotropinoma. Tylko ekspresja Ki-67 oraz TOPO 2A korelowała z wielkością guza i jego inwazyjnością. (*Endokrynol Pol 2018; 69 (2): 182–189*)

Słowa kluczowe: gruczoł przysadki, markery proliferacji, Ki-67, TOPO 2A, AIP, VEGF

Introduction

Somatotropinomas form 15–20% of all pituitary adenomas (PAs). The estimated frequency of acromegaly is

50–70 cases/million [1, 2]. Although mainly benign, PAs with invasive growth (30–45%), recurrence, resistance to multimodal treatment, and “atypical” morphologic features, are considered as being clinically aggressive [3, 4].

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However, their invasiveness is not clearly defined in WHO 2004 classification, which distinguishes only between typical and atypical adenomas (characterised by invasive growth, increased mitotic index, Ki-67 > 3%, and extensive nuclear expression of p53), and carcinomas [5–7].

Markers of proliferation and angiogenesis, over-expression of which was found in PAs, are potential indicators of tumour behaviour. Apart from the well-documented Ki-67, TOPO 2A (topoisomerase 2A), AIP (Aryl Hydrocarbon Receptor-Interacting Protein), and VEGF (Vascular Endothelial Growth Factor) have been suggested.

Ki-67 is a nuclear antigen expressed in all cell cycle phases, except G0; therefore, it is recognised as a proliferation marker. In pituitary tumours, Ki-67 indices may vary between < 1% and 23%, and do not correlate with tumour size [8]. A high Ki-67 index, as a marker of proliferation in atypical adenomas, does not appear to be reliable in predicting tumour recurrence [7–9].

TOPO 2A is one of the key enzymes in DNA replication and cell division [10], indicating rapid cell proliferation in many tumours [11–13]. In PAs, strong correlation between MIB1 and TOPO 2A expression was found [13].

AIP, encoded by a suppressor gene, is present in the nucleus and cytoplasm of many human tissues, e.g. heart, brain, or tonsils. AIP is involved in many, but not fully understood, cellular pathways [15]. Germline mutations of AIP are associated with familial isolated pituitary adenoma (FIPA) secreting hGH and/or PRL, found in young patients [16]. There are no somatic AIP mutations [17]. Mutant AIP loses the ability to reduce cell proliferation. AIP expression has also been shown to be a predictor of response to octreotide LAR (SSLAR) therapy, independently of somatostatin receptor type 2 (SSTR2) expression [18].

VEGF is an angiogenic factor expressed in the cytoplasm and nucleus in many solid tumours, stimulating cell proliferation and migration, increasing vascular permeability, being associated with the development of metastases and poor prognosis [19]. However, the role of angiogenesis in the pituitary is not clear as PAs are less vascularised compared to other tumours and to the normal pituitary gland. Higher VEGF expression in PAs than in normal tissue has been shown [20]; however, the results are not conclusive as different methods were used. VEGF is presumed to be a biomarker of pituitary neoplasms [8, 16].

Prediction of pituitary tumour behaviour and disease recurrence after neurosurgery is still a challenge. To treat patients more effectively, optimal and standardised markers of invasiveness and proliferation of PAs are being sought. Selection of patients with aggressive tumours based on immunohistochemistry

and molecular studies is essential for individualised treatment. Therefore, in this work we studied the correlation of selected markers with tumour invasiveness, progression, and recurrence.

Materials and methods

We retrospectively studied clinical, histopathology, and immunohistochemistry records of 31 patients of our Clinic of Endocrinology, who underwent pituitary surgery (transsphenoidal resection and/or craniotomy, with 42% of patients successfully operated), between 1997 and 2008, followed up for 24 and 36 months. Eleven patients were treated with SSLAR before surgery. The PA diagnosis was based on clinical, biochemical, and radiological data, confirmed by surgery and histopathology examinations. Table I presents our patient characteristics.

Tumour size, defined as its largest dimension and its relation to adjacent tissues, was evaluated by 1.5 T MR images obtained prior to surgery, 12 months, and 24–36 months after surgery. Tumour invasiveness was defined according to radiological criteria of Knosp [21] and Zada [22] and surgeon descriptions, as given in patient records. Recurrence was defined as a new lesion or enlargement of residual pituitary adenoma in MRI after surgery.

The surgical specimens were routinely processed, fixed in neutral buffered formalin, embedded in paraffin, cut into 4 µm-thick slides, and stained with H-E. Specific primary antibodies (Dako, Glostrup, Denmark) were used against pituitary hormones: ACTH, GH, PRL, TSH, LH, and FSH.

For immunohistochemistry (IHC), sections were incubated with the following antibodies: primary mouse monoclonal antibody Ki-67, clone MIB-1 antibody (Dako, M7240, in optimum dilution 1:25), Novocastra primary mouse monoclonal antibody Topoisomerase 2A, clone 3F6 (NCL-TOPO 2A, Leica Biosystems, 1:100), primary mouse monoclonal antibody AIP, clone LS-B227 (LSBio, 1:300), and primary rabbit monoclonal antibody VEGF, clone SP28 (Thermo Fisher Scientific, 1:100). Manufacturer's recommendations were followed for each antibody. Control of specificity of the primary antibody, and positive/negative control tests were performed according to the manufacturer's instructions.

Immunostained sections were evaluated by optical microscopy (NIKON OPTISHOT-2). Ten fields were selected in regions of highest concentration of positive cells and examined at 400x magnification. A total of at least 2000 cells were viewed in each section. The labelling index (LI) was evaluated as the percentage of positive cells — stained nuclei for Ki-67 and TOPO 2A, or stained cytoplasm for AIP and VEGF.

The study was approved by the Local Bioethics Committee of the Jagiellonian University Medical College (KBET/84/B/2012).

Basic statistical and comparative analyses appropriate to the distributions of data were performed. Kolmogorov-Smirnov, Mann-Whitney, Kruskal-Wallis, analysis of variance, T-test, Wilcoxon test, and Fischer exact tests were applied. Relative risk (RR) was calculated based on χ^2 frequency tables. Linear regression was applied to evaluate the correlation of normally distributed variables ($n \geq 30$). Otherwise, non-parametric Spearman rank correlation test was applied. For Kaplan-Meier (time-to-incidence plot with 36 months as the last follow-up point) and receiver operating characteristic (ROC) plots, GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego California, United States) was used. To establish predictors of recurrence and pituitary tumour advancement, Cox multiple regression and logistic regression were generated using the Statsdirect version 2.0 for Windows (Statsdirect Ltd., Cheshire, United Kingdom). P values of 0.05 or less were considered as being statistically significant.

Results

Median concentrations of hGH and IGF-1 decreased significantly after surgery ($p < 0.05$) (Table I). No correlation was found between hGH and IGF-1 concentration and tumour size prior to and after surgery ($p > 0.05$).

The percentage of adenomas ($n = 31$) with positive immunostaining for Ki-67, TOPO 2A, AIP, and VEGF was 24 (77.4%), 27 (87.1%), 26 (83.9%), and 27 (87.1%), respectively (Figure 1).

Mean and median values of studied indices are given in Table II.

In micro- and macroadenomas, expression of Ki-67 differed significantly with median values of 0.00% [IQR = 1.32] and 1.45% [IQR = 1.81], respectively ($p = 0.0155$) (Figure 2). Expression of Ki-67 exceeding 3% was observed in 3/31 tumours (9.7%). The maximum value of Ki-67 observed in one macroadenoma was 4.94%. Similarly, there was a significant difference between TOPO 2A expression in micro- and macroadenomas, with mean values of $0.71 \pm 0.77\%$ and $2.22 \pm 1.50\%$, respectively ($p = 0.001$) (Figure 2). However, there was no difference in AIP and VEGF expression.

No correlation between age or gender and Ki-67, TOPO 2A, AIP, and VEGF expression was found ($p > 0.05$). However, there was a significant correlation between tumour size and expression of Ki-67 ($r = 0.42$, $p = 0.025$) and TOPO 2A ($r = 0.53$, $p = 0.003$). No correlation of AIP and VEGF with tumour size was noted. Interestingly, a correlation between Ki-67 and TOPO 2A indices was observed ($r = 0.46$, $p = 0.01$).

Table I. Patients' characteristics
Tabela I. Charakterystyka pacjentów

General data	Age: mean \pm SD [y]	43 \pm 14.3
	Gender: Female/male (%)	20 (64.5%)/11 (35.5%)
	Microadenoma (%)	8 (26.0%)
	Macroadenoma (%)	23 (74.0%)
	Size of tumour [mm]	16 [14.4]/22.2 \pm 20.7
	Median [IQR]/mean \pm SD	
	Local invasion of tumor	18 (58.1%)
	Compression/dislocation or modeling of the optic chiasm (%)	10 (32%)
	Sella turcica destruction	5 (16.1%)
	Cavernous sinus penetration	15 (48.4%)
	Suprasellar propagation	14 (45.1%)
	Complete surgery	13/31 (42%)
Radiological progression/recurrence in MRI (patients %)	after 12 months	6/31 (19.35%)
	after 36 months	5/31 (16.13%)
Immunohistochemical findings* (n = 31)	Hormonal positivity	29 (93.7%)
	NCA	1 (3.2%)
	GH positive	27 (87.1%)
	PRL positive	18 (58.1%)
Number of patients(%)	ACTH positive	7 (22.6%)
	TSH positive	7 (22.6%)
	FSH positive	4 (12.9%)
	LH positive	2 (6.5%)
	Plurihormonal	12 (38.7%)
	Histopathological features of malignancy*	4 (12.9%)
Hormones before surgery	hGH median [IQR] (min-max) [ng/ml]	18.5 [19.8]/(2.8–170.0)
	IGF-1 median [IQR] (min-max) [ng/ml]	768.0 [610.2]/(174.9–1999.0)
	**ULN median (min-max)	2.23 (0.40–4.33)
Hormones after surgery	hGH median [IQR] (min-max) [ng/ml]	2.25[4.0]/(0.45–50.0)
	IGF-1 median [IQR] (min-max) [ng/ml]	267.0 [319.5]/(43.3–980.0)
	**ULN median (min-max)	0.65 (0.14–2.32)

*Pituitary adenomas were classified according to WHO 2004 classification

**ULN — upper limit of normal IGF-1 concentration

n = 31, y — years

Local invasiveness (cavernous sinus penetration, optic chiasm compression, or suprasellar extension) occurred in 18/31 patients (58.1%). There was a significant

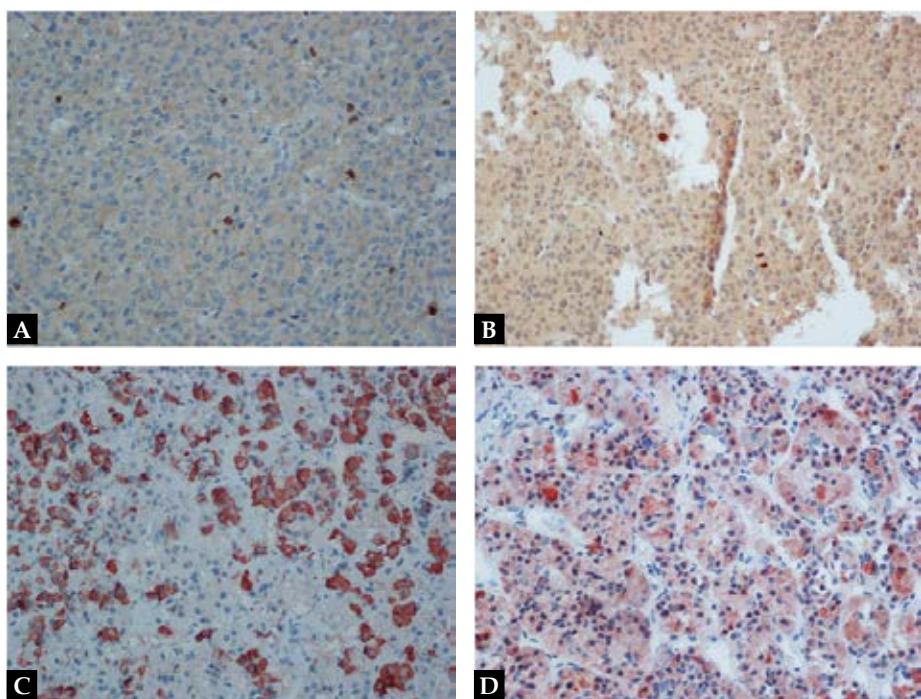


Figure 1. Immunohistochemical staining for studied markers of proliferation and invasion in pituitary adenomas, magnitude 200x: **A.** Ki-67 nuclear staining; **B.** TOPO 2A — nuclear staining; **C.** AIP diffuse cytoplasmic immunostaining; **D.** VEGF — diffuse cytoplasmic immunostaining

Rycina 1. Ekspresja badanych markerów proliferacji i inwazyjności w gruczołakach przysadki — barwienie immunohistochemiczne, powiększenie mikroskopowe 200x: **A.** Ki-67 barwienie jądrowe; **B.** TOPO 2A — barwienie jądrowe; **C.** AIP — rozlane barwienie cytoplazmatyczne; **D.** VEGF — rozlane barwienie cytoplazmatyczne

Table II. Mean and median values of labelling indices of the studied markers

Tabela II. Mediany i wartości średnie indeksów barwienia dla badanych markerów

Parameter	Mean value ± SD	Median value [IQR]
Ki-67	1.5% ± 1.3	1.2% [IQR = 2.2]
TOPO 2A	1.9% ± 1.5	1.5% [IQR = 1.6]
AIP	26.1 ± 21.3	21.2 % [IQR = 20.1]
VEGF	21.4% ± 15.9	20.4% [IQR = 15.4]

difference between mean values of Ki-67 index in invasive vs. non-invasive tumours: $1.85 \pm 1.33\%$ vs. $0.95 \pm 1.07\%$, respectively ($p = 0.024$) (Figure 3). Similarly, there was a significant difference between mean TOPO 2A index values in invasive vs. non-invasive tumours: $2.19 \pm 1.63\%$ vs. $1.45 \pm 1.23\%$, respectively ($p = 0.011$) (Figure 3).

Local recurrence/progression of the tumour, based on MRI evaluation after 12 and 36 months, occurred in 11 patients (35.5%). There was no difference in median values of the studied markers between recurrent and non-recurrent adenomas.

In ROC curve analysis, no cut-off point values were statistically significant for any marker. Therefore, we

arbitrarily selected the following cut-off points: Ki-67 — 1%, TOPO 2A — 1%, VEGF — 10%, and AIP — 15%. On the plot of cumulated hazard, we tested whether expression of these markers above such cut-off points affected the frequency of disease recurrence. No significant difference was found for any marker. Using Cox multiple regression analysis, we calculated the relative risk (RR) of tumour recurrence depending on labelling index — no significant risk was found above these thresholds, except for AIP — above 15%, with RR = 3.85 (95% CI 71.96–98.93).

In 11 patients treated preoperatively with SSLAR (20–30 mg monthly) no expression of any of the markers was significantly affected. However, LI of VEGF was markedly lower in SSLAR-treated patients: 9.6% [8.79] vs. non-treated: 23.78% [23.24] ($p = 0.075$) (Table III).

Discussion

In the search for new markers of invasiveness, proliferation and angiogenesis in pituitary adenomas based on molecular mechanisms involved in uncontrolled cell proliferation and tumour growth, of particular interest are biomarkers that reliably predict the biological behaviour, allowing for targeted therapies.

We investigated the expression of Ki-67, TOPO 2A, AIP, and VEGF as proliferation markers of somatotropinomas

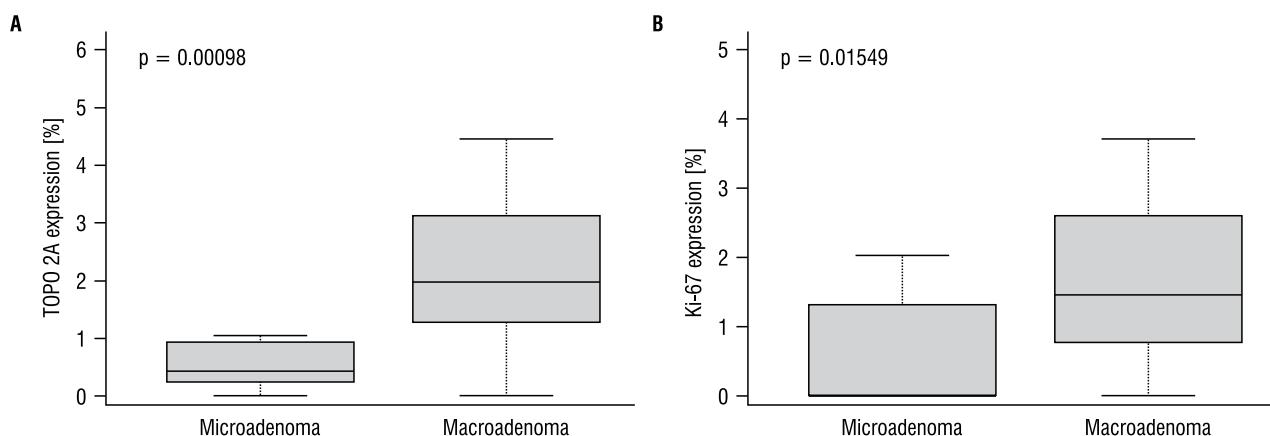


Figure 2A. Expression of TOPO 2A in micro- and macro-adenomas secreting hGH (mean \pm SD of LI); **B.** Expression of Ki-67 in micro- and macro-adenomas secreting hGH (median [IQR] of LI)

Rycina 2A. Ekspresja TOPO 2A w mikro- i makrogruczołakach przysadki wydzielających hGH (wartości średnie \pm SD LI); **B.** Ekspresja Ki-67 w mikro- i makrogruczołakach przysadki wydzielających hGH (mediany [IQR] LI)

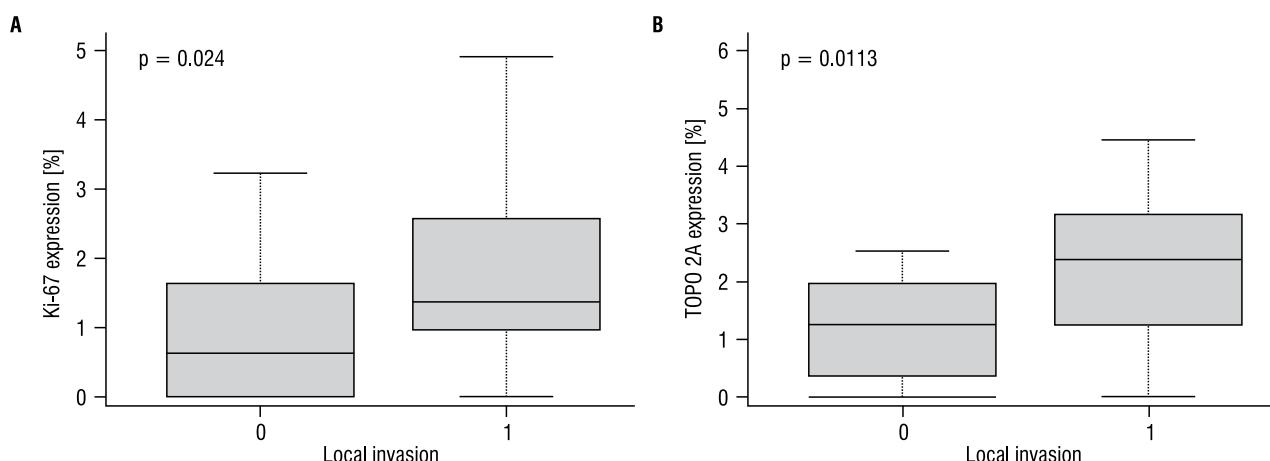


Figure 3A. Expression of Ki-67 in non-invasive and invasive somatotropinomas (mean \pm SD of LI); **B.** Expression of TOPO 2A in non-invasive and invasive somatotropinomas (mean \pm SD of LI); 0 — non-invasive somatotropinomas; 1 — invasive somatotropinomas

Rycina 3A. Ekspresja Ki-67 w nieinwazyjnych i inwazyjnych gruczołakach przysadki wydzielających hGH (wartości średnie \pm SD); **B.** Ekspresja TOPO 2A w nieinwazyjnych i inwazyjnych gruczołakach przysadki wydzielających hGH (wartości średnie \pm SD); 0 — nieinwazyjne somatotropinoma; 1 — inwazyjne somatotropinoma

Table III. Expression of studied markers (LI) in patients treated ($n = 11$ patients) and non-treated with SSLAR prior to surgery of pituitary adenoma

Tabela III. Ekspresja badanych markerów (LI) w grupie pacjentów leczonych ($n = 11$) i nialeczonych SSLAR przed operacją gruczołaka przysadki

Marker	Non-treated median [IQR] [%]	Treated median [IQR] [%]	P*
Ki-67	1.3% [2.25]	2.03% [2.57]	0.78
TOPO 2A	1.49% [1.9]	1.27% [2.11]	0.39
AIP	23.3% [32.4]	18.03% [19.76]	0.56
VEGF	23.78% [23.24]	9.6% [8.79]	0.075

in patients who underwent neurosurgery. We evaluated the relationship between these markers and patient demographic data and between tumour features to establish their prognostic value and indication for reoperation or radiotherapy. We also analysed the effect of preoperative treatment with SSLAR on the expression of the studied markers.

Expression of investigated markers

The percentage of adenomas with positive immunostaining for Ki-67 in the study group was over 77% as compared to 88% or 100% as reported by other authors [23, 24]. In our study somatotropinomas presented generally with Ki-67 index < 3%, and high variation (range

0–4.94%), with values exceeding 3% in only three atypical tumours. In a group of predominantly non-functioning adenomas, Ki-67 indices ranged from 1% to 4.8% [24] and from 1.4% to 5.2% in somatotropinomas [25].

TOPO 2A-positive immunostaining in our somatotropinomas was higher than that in patients with different types of PAs [11, 13], where values of the TOPO 2A index ranged from 0% to 3.5%, with a median value of about 0.7% [13, 26], against our median LI of 1.5%.

AIP expression in somatotropinomas is ambiguous. AIP mutations were detected in 2–4% of young acromegalic patients with sporadic microadenoma and macroadenoma [17]. In our sporadic somatotropinomas AIP was positive in over 80% of tumours, which is in line with other studies [27], while Kasuki et al. found low expression in only about 50% of somatotropinomas [18].

VEGF immunostaining in normal pituitary glands was higher than in adenomas, with greater expression in pituitary carcinomas in relation to adenomas [28], while the highest expression of VEGF was found in non-functioning and GH-producing adenomas. On the other hand, in a RT-PCR study of different subtypes of pituitary adenomas, somatotropinomas showed lowest VEGF expression [29, 30]. In IHC, expression of VEGF was decreased in somatotropinomas, against normal tissue [31].

We note the difficulty of comparing our AIP and VEGF results with those of other authors who analysed different types of PAs using different evaluation methods. In our view, the particular value of our investigation is in its relatively large and homogeneous group of GH-secreting adenomas.

Age and gender

In our group of somatotropinomas we found no correlation between age and gender, and values of the studied markers. In other studies of GH-secreting adenomas, Ki-67 index depended on gender but not on patient age [32]. Results concerning TOPO 2A expression are generally inconclusive: Vidal et al. [11] found negative correlation between TOPO 2A expression and patient age and no correlation with patient gender, while elsewhere TOPO 2A expression did not depend on patient age [13, 14]. Age and gender and adenoma type did not affect AIP [18] and VEGF expression [31].

Tumour size

We found clear correlation between values of Ki-67 and TOPO 2A indices and tumour size, the difference between values of Ki-67 and TOPO 2A in micro- and macro-adenomas being statistically significant. However, lack of correlation between Ki-67 expression and tumour size was also reported [8]. Elsewhere, higher TOPO 2A expression was shown in non-functioning macroadenomas, which correlated with tumour size [13],

while no correlation between TOPO 2A expression and tumour size [14], or even negative correlation [11] were also observed. A low AIP score was found in large somatotropinomas [33]. No correlation of VEGF expression with tumour size was stated [29, 31], which our study confirms.

Invasiveness

Local invasion and infiltration of the adjacent structures, postoperative regrowth or persistence of hormonal function are potential indicators of PA aggressiveness [11, 13, 21, 22]. In our study Ki-67 and TOPO 2A indices were significantly higher in invasive vs. non-invasive somatotropinomas. Determination of TOPO 2A activity enabled a group of invasive pituitary adenomas to be distinguished [11, 13]. However, no advantage of TOPO 2A over Ki-67 was stated [11]. In the work of Kasuki et al. [34] the range of Ki-67 index in invasive somatotropinomas (0–20.6%) was much wider than that in our group, with a 2.3% cut-off point on their ROC curve. Other investigators found no correlation between Ki-67 index and somatotropinoma invasiveness [35, 36]. Nevertheless, the opinion prevails that radiological grading, IHC classification, and Ki-67 as prognostic markers are important in evaluating the true nature of atypical pituitary adenomas [4, 5, 7, 16, 23, 37].

In our somatotropinomas there was no significant difference between mean values of AIP and VEGF indices in invasive vs. non-invasive tumours. In some 50% of sporadic adenomas, low intrinsic AIP expression, predominant invasiveness, and poor response to somatostatin analogues (SSA) are observed [27, 33, 34, 38], especially in patients with confirmed AIP mutation [27]. While in somatotropinomas AIP was claimed to be a better marker of invasiveness than Ki-67 and p53 [34], its expression varied widely in our study and elsewhere [27].

In agreement with Kurosaki et al. [31], we found no difference in VEGF expression between invasive and non-invasive tumours, as opposed to the suggested VEGF LI above 25% as a cut-off value for invasive somatotropinomas [36]. Higher VEGF expression was seen in invasive non-functioning giant adenomas and tumours with extrasellar growth [25, 29], in contrast to low VEGF expression in patients, 70% of whom achieved remission [35]. We may thus presume that VEGF plays an indirect role in the development of somatotropinomas, being an independent stimulator of angiogenic growth and progression in GH-secreting adenomas.

Tumour recurrence

In our study, no differences between median values of relevant indices for any marker were found, nor could any cut-off points be established for recurrent and

non-recurrent adenomas. Therefore, in our cohort, no relation between these parameters and clinical outcome could be demonstrated.

However, we noted after 12 months of observation that recurrences occurred in all and in almost all adenomas in which Ki-67 and TOPO 2A indices exceeded the arbitrarily chosen cut-off points of 1%.

In our group of somatotropinomas, in only three patients (9.7%) did Ki-67 index exceed 3%, this patient number not being statistically significant. The role of Ki-67 as a marker of recurrence in pituitary tumours is not established. In small and non-homogenous patient groups with non-functioning PAs and somatotropinomas, expression of Ki-67 was not related to tumour recurrence nor to local invasion [23, 24]. Magagna-Poveda et al. [39] confirmed earlier studies that only the MIB-1 proliferation index was related to tumour recurrence. In their study of 35 giant pituitary adenomas, 60% of patients with $\text{Ki-67} > 3\%$ experienced tumour recurrence, as compared to patients with $\text{Ki-67} < 3\%$, in whom the recurrence rate was only 6.7%. Grupetta et al. [8] confirmed that high Ki-67 predicts tumour regrowth.

Our rate of recurrence is similar to that found by other authors [8, 13, 39], although in their case non-homogenous groups of different pituitary adenomas including GH-secreting tumours were evaluated.

Studies of the relation between tumour recurrence and expression of VEGF and AIP based on IHC are sparse and contradictory. A low level of VEGF detected PAs with higher rates of regrowth [8]. A low level of AIP was associated with higher proliferative activity, predicting recurrence in somatotropinomas [40].

In our work, the dependence of RR of tumour recurrence on the values of markers studied was not significant above any of the arbitrarily chosen cut-off points, except in the case of AIP above 15%, indicating that the chance of recurrence is over three times higher.

Pre-surgical SSLAR treatment

In our somatotropinomas, SSLAR treatment did not significantly affect the expression of any of the studied markers. However, VEGF expression was markedly lower in the treated vs. non-treated groups of patients.

Somatostatin analogues in patients with acromegaly inhibit TOPO 2A expression, providing molecular evidence of their effectiveness [11, 13]. SSA modulate AIP in somatotropinomas, increasing the number of AIP-positive cells in immunostaining after lanreotide treatment, compared with patients treated by surgery only [41].

The AIP score was up-regulated in SSA-treated tumours with better preoperative response such as IGF-1 decrease or tumour shrinkage [42], suggesting that AIP is involved in the aggressiveness of sporadic somatotropinomas [33]. Therefore, AIP expression in

sporadic somatotropinomas might be a predictor of the response to SSA therapy independently of SSTR2 expression [18].

In patients with invasive adenomas pre-treated with SSA before surgery, a decrease in VEGF staining was observed [28, 31, 36], as well as lower expression of VEGF mRNA [29], suggesting that SSA may inhibit angiogenesis through down-regulation of VEGF. However, studies on SSA modulation of VEGF expression are few and inconclusive [29].

Ki-67 and TOPO 2A correlation

In our group of somatotropinomas, we found correlation between Ki-67 and TOPO 2A, in line with Wolfsberger et al. [14], who found a strong correlation between MIB-1 and TOPO 2A expression in PAs, unlike Vidal et al. [11]. In contradiction to our study, a significant negative correlation between AIP score and Ki-67 index was also observed [33].

Conclusions

Markers of proliferation: Ki-67, TOPO 2A, AIP, and VEGF were expressed in over 70% of all somatotropinomas. Only Ki-67 and TOPO 2A expression was related to tumour size. Ki-67 and TOPO 2A expression was found to correlate with tumour invasiveness.

Since almost all somatotropinomas expressed Ki-67, TOPO 2A, AIP, and VEGF, these markers are potential targets for non-invasive treatment.

Declaration of interests

The authors have no potential conflicts of interest to declare.

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Autorship and contributions

Agata Baldys-Waligorska: conception of statutory grant, design of study, writing of the manuscript, reference search
Iga Wierzbicka: methods, investigation

Grzegorz Sokołowski: analysis of data, statistics, illustrations

Dariusz Adamek: methods, investigation

Filip Golkowski: analysis of data, reference search, editing

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