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**Article ID:** AOP_14_065

**ISSN:** 1897-9483

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**Article type:** Original article

**Submitted:** April 15, 2014

**Accepted:** August 13, 2014

**Published online:** September 3, 2014

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Topoisomerase 2 alpha as a prognostic factor in pituitary tumors

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Short title: Topoisomerase 2 alpha in pituitary tumors

Word count of the full article: 3976

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Abstract

**Introduction:** In pituitary tumors markers of proliferation and progression essential for treatment and follow-up are searched

**Objectives:** We studied topoisomerase 2 alpha (Topo2A) expression in different types of pituitary adenomas in order to evaluate its prognostic potential in patients with hypophyseal tumors.

**Patients and methods:** In a retrospective study of 60 patients of mean age 46.7±17.6 years who underwent pituitary tumor surgery, expression of Topo2A, by immunohistochemistry, was analysed quantitatively with respect to histopathology, tumor features, clinical symptoms, MR imaging and post-operative recurrence/progression of disease.

**Results:** Topo2A was expressed in 73% of (44/60) pituitary adenoma. The highest Topo2A indices were observed in ACTH-secreting pituitary tumors (median:1.13% [0.37-1.21]), followed by silent-ACTH tumors (0.94%[0.89-1.0]), and hormone immunonegative adenomas (0.8%[0.65-1.55]). No differences in expression with respect to patient age or gender were observed. Statistically significant relations were found between Topo2A index and tumor size, its invasiveness, pathological ocular tests and recurrence of tumor growth in postoperative observation. In patients with Topo2A index >1% the relative risk of tumor recurrence is higher by a factor of 3.5 (95%CI:1.8-6.9), \(P<0.001\). After pre-treatment acromegaly patients with somatostatin analogues decrease in median Topo2A expression was observed, compared with untreated patients (0.0% [0.0-0.22] vs 0.71%[0.17-1.0], \(P<0.05\).

**Conclusions:** In our study group, Topo2A index exceeding 1% was found to be a prognostic factor for recurrence/progression of tumors, especially in patients with hormonally inactive adenomas, to be selected for intensive postoperative treatment. In acromegaly, application of
somatostatin analogues inhibits Topo2A expression, providing molecular evidence of the effectiveness of these analogues.

**Key words:** aggressive pituitary tumor, biomarker, topoisomerase 2 alpha
Introduction

Following the 2004 WHO (World Health Organization) classification of pituitary tumors [1-4] where the concept of atypical adenoma was introduced, the Ki-67 antigen and p53 immunoreactivity are among the most frequently evaluated markers in pituitary adenomas. The new prognostic clinico-pathological classification of pituitary adenomas proposed by Trouillas et al. [5] also uses these markers of cell cycle for tumor grading.

The Ki-67 antigen, present in all active phases of mitosis, is absent in the G0 phase. A high Ki-67 index correlates well with tumor invasiveness and its recurrence following surgical treatment [6,7], however, low Ki-67 indices, comparable with those typical of non-invasive tumors, are quite frequently observed in aggressively progressing adenomas [8,9].

Despite considerable progress in understanding the pathogenesis of pituitary adenomas, no single marker has been found to predict aggressive behavior of pituitary adenomas independently. Therefore other specific markers of pituitary adenoma proliferation and angiogenesis, including microRNAs, are under investigation [10,11].

Topoisomerase 2 alpha (Topo2A) has been established as one of the key enzymes in DNA replication and cell division [12] indicating cell proliferation activity in many tumors. Topo2A is also the target for several cytostatic drugs [13,14].

Determination of Topo2A activity enabled the group of invasive pituitary adenomas to be distinguished [15,16]. In pituitary adenomas, Wolfsberg et al. [17] found a strong correlation between MIB-1 and Topo2A expression.

The aim of our work was to investigate whether topoisomerase 2 alpha expression (as based on immunohistochemical staining) could serve as a prognostic factor in the treatment of
patients with pituitary adenoma. In our study we investigated retrospectively the correlation of Topo2A *labelling index* (LI) with demographic, clinical and imaging data of these patients.

**Materials and methods**

A group of 60 patients with pituitary adenoma, (patient characteristics - Table 1), were admitted to our Clinic of Endocrinology after pituitary surgery in the years 2003-2006. Each patient was followed-up for 48 months after surgery.

All patients were operated by the same team of neurosurgeons. The tumors were surgically removed by transsphenoidal resection. The final diagnosis of these patients was based on demographic and clinical data, results of post-surgical specimen histopathology and evaluation of magnetic resonance imaging (MRI) retrieved retrospectively from their medical records (Table 1).

The study was approved by the Bioethical Committee of the Jagiellonian University Medical College.

Fundus abnormalities and visual field defects were assessed retrospectively from patients’ medical records.

Tumor size, defined as its largest dimension, sella turcica destruction, penetration into cavernous or sphenoid sinus, optic chiasm compression (Table 1) were evaluated from 1.5 T MR images obtained prior to, and 3-6, 12, 24, 36 and 48 months following surgery. Routine T1-weighted spin-echo sequences, before and after administration of 0.1 mmol gadolinium chelate were obtained. All MR images were evaluated by the same radiologist.
Tumor invasiveness was assessed according to radiological criteria of Knosp et al. [18] and Zada et al. [19] and from surgeon’s descriptions in the patient records. Tumor recurrence/progression was defined as regrowth (enlargement) of residual pituitary adenoma after surgery.

Patients with prolactinoma who required pharmacological treatment (6/13) received dopamine agonist (bromocriptine) for 3-6 months. Patients with acromegaly (8/16) were treated with somatostatin analogue (octreotide LAR) 6-12 months prior to surgery. Patients with pituitary insufficiency received appropriate hormone replacement. Prior to surgery no patient was diagnosed with diabetes insipidus. Recurrence of hormonal secretion was considered in patients who did not fulfil generally accepted criteria of cure after surgery [20-23].

Surgically obtained specimens of pituitary adenomas were stained with hematoxyline and eosine. Specific primary antibodies against pituitary hormones: ACTH, GH, PRL, TSH, LH, FSH (Dako, Glostrup, Denmark) were used. All samples were evaluated by the same pathologist. Tumors with atypical morphology features such as increased pleomorphism, elevated mitotic activity, suggestive of invasive growth, were defined as “more aggressive”.

Pituitary adenoma specimens were classified according to WHO criteria. Tumors with no expression of ACTH, GH, PRL, TSH, LH or FSH were classified as hormone immunonegative adenomas [1].

Immunohistochemical staining for Topo2A with monoclonal IgG-class antibodies directed against C-terminal domain of human Topoisomerase (NCL-Topo 2 alpha by Novocastra) was performed in optimum dilution (1:30). The antigen was retrieved by microwave treatment at 95°C in citrate buffer, pH=6.0. Overnight incubation with primary antiserum (NCL-Topo 2 alpha) at refrigerator temperature was followed by incubation with secondary biotinylated antibody for 30 minutes. Next, avidin-biotin complex horseradish peroxidase (ABC-HRP) (30
minutes) with DAB (diaminobenzidine tetrahydrochloride) as chromogen, was applied for up to 8 minutes under microscope control, followed by counterstaining with hematoxylin (Hematoxylin, Mayer).

Control of specificity of the primary antibody, and positive and negative control tests were performed according to manufacturer’s instructions. Sections of anaplastic gastric cancer served as positive control. Substitution of primary antibody with phosphate buffered saline pH=7.4 served as negative control. Topo2A-immuno-stained sections were evaluated by optical microscopy (NIKON OPTISHOT-2) at 400 x magnification. The Topo2A labelling index was evaluated as the percentage of positively stained cells with respect to the total of at least 2000 cells viewed in each section.

Basic statistics and comparative analysis appropriate to the distributions of data points, were applied. Kolmogorov-Smirnov, U-Mann-Whitney, Kruskal-Wallis, Anova and Fischer’s exact tests were used as appropriate. Relative risk (RR) and odds ratio (OR) were calculated based on chi² frequency tables. Linear regression was applied to evaluate the degree of correlation of n≥30 normally distributed variables. Otherwise, the non-parametric Spearman rank correlation test was performed.

In Kaplan-Meier graphs, time-to-incidence was plotted, with 48 months as the last observation period. For Kaplan-Meier and ROC (Receiver Operating Characteristic) plots statistic tests were generated using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego CA, USA).

To establish predictors of recurrence and pituitary tumor advancement Cox multiple regression and logistic regression statistic tests were generated using the Statsdirect version 2.0 for Windows (Statsdirect Ltd., Cheshire, UK). The level of statistical significance used in this study was $P \leq 0.05$. 
Results

Occurrence of hormone immune-positive staining was established in 41/60 (68.3%) cases, as based on immunohistochemistry records. 19/60 tumors with no expression of ACTH, GH, PRL, TSH, LH nor FSH, were classified as hormone immunonegative adenomas [1].

Of all 60 patients 35 (58,3%) had functioning tumors, while 25 (41.7%) had non-functioning tumors, of which 4 cases of gonadotropin-positive adenomas and 2 ACTH-positive tumors were found in histopathology.

Finally, 13 (21.7%) patients were classified as prolactinoma, 16 (26.7%) as acromegaly, 4 (6.7%) with Cushing disease, 5 (8.3%) with gonadotropinoma, 1 (1.7%) with thyrotropinoma, and 2 (3.4%) as patients with silent-ACTH adenoma. Expression of more than one hormone was found in some adenomas (Table 1).

In six cases (10%) of the examined adenomas, polymorphism of cell nuclei and presence of mitotic figures was demonstrated, suggestive of aggressive course and more invasive growth [1].

Between the 3\textsuperscript{rd} and 48\textsuperscript{th} months of observation after surgery, recurrence or progression of the pituitary tumor (diagnosed by MRI or by hormonal tests), was stated in 22/60 patients (36.7 %).

Topo2A expression was stated in 44/60 (73%) cases of pituitary adenoma. Topo2A immunoreactivity was present in cell nuclei additionally stained with hematoxylin (Figure 1).

Values of Topo2A labelling index ranged between 0% and 3.5%, with a median value of 0.71% [0.0-1.1].
No differences in Topo2A expression with respect to patient age or gender (female 0.71\% [0.0-1.18] vs male 0.67\% [0.13-1.04], \( P > 0.05 \)), were observed.

The highest median values of Topo2A index (1.13\% [0.37-1.21]) were observed in ACTH-secreting pituitary tumors leading to Cushing syndrome, followed by silent-ACTH tumors (0.94\% [0.89-1.0]), and hormone immunonegative adenomas (0.8\% [0.65-1.55]). The median values of Topo2A indices in each type of tumor did not differ significantly (Figure 2).

In patients with hormone immunonegative adenomas the median value of Topo2A index (0.8\% [0.65-1.55]) was significantly higher than that in patients with hormone secreting tumors (0.41\% [0.0-1.04]), \( P < 0.05 \).

In patients (32/60, 53.3\%) with documented visual defects the median value of Topo2A labelling index (0.89\% [0.18-1.54]) was higher, compared with patients without such symptoms (0.43\% [0.0-0.92]), \( P < 0.05 \).

In patients with visual field defect the median value of Topo2A index (0.89\% [0.15-1.55]) was higher than that in patients with normal visual field (0.5\% [0.0-0.97]), \( P < 0.05 \).

The mean value of largest tumor dimension was 25.1±17.6 mm (Table 1). In pituitary adenomas the value of Topo2A index correlated with tumor size (\( P < 0.05 \), \( r = 0.31 \)).

Significantly higher values of Topo2A index were observed in macroadenomas as compare to microadenomas (0.73\% [0.13-1.2] vs 0.19\% [0.0-0.97], \( P < 0.05 \), respectively.

Significant differences were found between median Topo2A indices in patients with invasive tumors (Table 1) compared with patients where no such features were observed 0.89\%[0.43-1.43] vs 0.0\%[0.0-0.84], \( P < 0.001 \) (Figure 3).
Applying the logistic regression model we found that Topo2A labelling index > 1% in hormone immunonegative adenomas implies the highest chance of development of adenomas penetrating cavernous sinus, odds ratio OR=0.23 (95% CI: 0.07 – 0.80, \( P < 0.02 \)).

Compared with other patients, the Topo2A expression in patients with local tumor recurrence or with regrowth of the post-surgical adenoma residue, as stated by MRI, was significantly higher (1.12% [0.7-1.8] vs 0.38% [0.0-0.88], \( P < 0.001 \)).

Applying Kaplan-Meier plots for groups of patients in whom values of Topo2A index were 1% or less, and those in whom this value exceeded 1%, significantly higher frequency of MRI-confirmed tumor recurrence was observed in the last group of patients (\( P < 0.001 \)) (Figure 4A). The same rule was found to apply in the case of patients with hormone immunonegative adenomas (\( P < 0.01 \)) (Figure 4B).

The relative risk (RR) of tumor recurrence in patients with expression of Topo2A is not statistically significant. However, for patients with Topo2A expression >1%, RR = 3.5 (95%CI: 1.8-6.9), \( p<0.001 \), i.e., the relative risk of tumor recurrence is 3.5 times higher.

Following Cox’s multiple regression analysis, only Topo2A expression >1% remained as an independent predictor of MRI-confirmed tumor recurrence or progression (Table 2).

At the cut off level of Topo2A index of 1%, Topo2A expression shows 63.6% sensitivity and 86.8% specificity in predicting tumor recurrence or progression. The large area under the ROC curve (area=0.76; 95% CI: 0.62-0.90; \( P < 0.001 \)) indicates the high significance of Topo2A expression in predicting tumor recurrence/progression (Figure 5).

Values of Topo2A expression were not significantly higher in patients with hormone secretion recurrence, compared with other patients (1.04% [0.0-1.21] vs 0.65% [0.0-1.01], \( P > 0.05 \)). Topo2A expression was found not to be related with the risk of hormone secretion
recurrence, RR=1.0 (95% CI: 0.4-2.7), \((P>0.05)\), also for patients with Topo2A index > 1%, RR=2.3 (95%CI: 0.9-5.4) \((P>0.05)\).

In acromegaly patients treated with octreotide LAR prior to surgery the mean Topo2A index was significantly lower (0.0% [0.0-0.22]) than that in patients not thus pre-treated (0.71% [0.17-1.0], \(P<0.05\).

In patients with prolactinoma, higher (but not statistically significant) values of Topo2A index were observed in patients not treated with bromocriptine prior to surgery, compared with patients pre-treated with dopamine agonist (0.9% [0.0-1.21] vs 0.0% [0.0-0.91]).

**Discussion**

In our study we investigated Topoisomerase 2 alpha as a proliferation marker in pituitary tumors of patients who underwent neurosurgery. We evaluated the relationship between Topo2A labelling index and demographic data and tumor features in the studied patients in order to establish its prognostic value to predict tumor invasiveness. We compared Topo2A expression against tumor progression/recurrence as indication for reoperation or radiotherapy. We also analyzed the effect on the Topo2A index of preoperative treatment with somatostatin analogues in acromegaly patients, or of dopaminergic agonists in prolactinoma patients. So far, no prognostic advantage of Topo2A over Ki-67 has been shown [16,17,24].

Pituitary tumors are usually benign and grow slowly, presenting no clinical manifestations, as evidenced by the disparity between the number of symptomatic adenomas against their number established on autopsy [25]. Local invasion and infiltration of adjacent structures, post-operative regrowth or persistence of hormonal function are potential indicators of aggressiveness [26-29]. In order to treat patients more effectively, rather than to await
confirmation of tumor recurrence by MR imaging, suitable markers of invasiveness and proliferation of pituitary adenomas are sought to select patients with atypical pituitary adenomas. In our group of 60 patients, 73% expressed Topo2A, against 76%, reported by Vidal et al. [16].

In agreement with Suzuki et al. [15] and Wolfsberger et al. [17], we found that Topo2A expression did not depend on age of our patients. However, Wolfsberger et al. [17] found that female gender is significantly associated with higher Topo2A index. Vidal et al. [16] found a negative correlation between Topo2A expression and patient age.

However, our patient group differs from those of the above authors, in the number of patients and the spectrum of adenomas analyzed.

In our study the expression of Topo2A was higher in macroadenomas and correlated with tumor size, in agreement with Suzuki et al. [15]. Wolfsberger et al. [17] found no dependence of Topo2A expression on tumor size, while Vidal et al. [16] suggested a negative correlation.

In hormone immunonegative adenomas, we found Topo2A expression to be higher, compared with other tumors. In particular, we observed high Topo2A indices in large adenomas compressing the optic chiasm and penetrating the cavernous sinus. Basing on results of MRI and hormonal tests, one may conclude that high values of Topo2A indices were seen predominantly in macroadenomas with no hormonal function.

Our results concur with those of Saeger et al. [24] who evaluated the expression of Ki-67, Topo2A i Cyclin D3 as proliferation markers in hormonally non-functioning pituitary adenomas.
In studies of Vidal et al. [16] or Wolfsberger et al. [17] involving larger groups of patients, highest values of Topo2A expression were observed in pituitary carcinomas, silent-ACTH adenomas, prolactinomas, somatotropinomas, and silent subtype 3 adenomas.

Our results differ from those cited above. The large dispersion of Topo2A index values found in our relatively small group of patients with diverse types of pituitary adenoma and no pituitary carcinoma cases may have affected the results of our statistical analysis.

We found the evaluation of visual field to be a significant clinical indicator of pituitary tumor invasiveness, correlating with Topo2A expression. Visual field impairment was observed in 65% of our macroadenoma patients, this being consistent with the correlation between Topo2A expression and tumor size. Visual field impairment in up to 74% of adenoma patients was stated by Thomas et al. [30].

Tumor recurrence or progression in patients undergoing transsphenoidal surgery is reported to occur in 30.8% [6] up to 46% [27] cases. Over a four-year period we observed recurrence or tumor progression in 36.7% of cases, as based on MRI, most likely due to large tumor sizes and to non-radical surgery.

We found significantly higher Topo2A expression in patients with tumor recurrence or progression, indicating that the predictive factor of tumor regrowth is represented by a high value of Topo2A index rather than by Topo2A expression only. While no markers for increased risk of recurrence have so far been established, our novel result is that Topo2A labelling index exceeding 1% predicts pituitary adenoma regrowth. From multiple regression analysis, we found Topo2A index greater than 1% to be an independent predictor of MRI-confirmed tumor recurrence or progression.
Thus, from our finding, patients in whom Topo2A index greater than 1% is stated, being at a higher risk of tumor recurrence, should be monitored more frequently and qualified for radiotherapy early.

Concurring with Suzuki et al. [15] and Vidal et al. [16], we have shown higher Topo2A index values in tumors which compress or infiltrate the sella turcica and cavernous sinus, than in tumors without such features.

Pre-operative treatment with somatostatin analogues in acromegaly patients results in a statistically significant decrease in Topo2A expression. Somatostatin receptors are not expressed exclusively in the pituitary cells. They are targeted for diagnostics and therapy in many diseases [31,32]. Application of somatostatin analogues, apart from achieving tumor shrinkage and better metabolic balance, also improves the efficiency of neurosurgery [33-35]. Low values of the Topo2A index in our patients treated with octreotide provide evidence, at the molecular level, of their effectiveness in controlling GH-producing adenomas. Vidal et al. [16] drew similar conclusions concerning Topo2A expression in patients treated pre-operatively with somatostatin analogues.

High Topo2A expression may be helpful in selecting adenohypophyseal tumors responsive to antiproliferative therapy. The possibility exists of introducing agents inhibiting expression of Topo2A in hormone immunonegative adenomas where pharmacotherapy options are currently limited [36-38]. In our study, these tumors showed the highest values of Topo2A expression and a high recurrence rate.

It is worth noting that somatostatin analogues and dopamine agonists have been shown to be efficient in some 12% and 27.6% of patients, respectively, in non-functioning pituitary adenomas, as summarized by Colao et al. [39]. We have shown this treatment to effectively decrease Topo2A expression. However, insufficient evidence-based data so far preclude any
clear recommendation that somatostatin analogues and dopamine agonists be applied in treating non-functioning pituitary adenomas.

Analysis of the results of immunocytochemistry and understanding subcellular mechanism that underlie pituitary tumor development will allow new tumor aggression markers and novel targeted therapies to be established.

Conclusions

1. Topo2A labelling index exceeding 1% was found to be a prognostic factor for recurrence/progression of tumors, especially in hormonally inactive adenomas.

2. Expression of Topo2A correlates with tumor size and degree of its invasiveness or of compression of anatomical structures around the sella turcica and may constitute a marker which enables patients with potentially aggressive pituitary adenomas to be selected for further therapy.

3. Use of somatostatin analogues in acromegaly patients inhibits the expression of Topo2A, providing molecular evidence of the effectiveness of these analogues.

Declaration of interest

The Authors declare no conflict of interest.

Funding

This work was supported by the Jagiellonian University Medical College statutory grant No BBN/CM-4103/411/2/2005/WL/NKL/83/L.
**Authors contribution statement**

Dr Malgorzata Trofimiuk and Dr Agata Baldys-Waligorska have contributed equally to this work.

**Acknowledgements**

The authors wish to thank Ms. Edyta Radwanska, M. Sc., for her excellent technical assistance. This work was supported by the Jagiellonian University Medical College statutory grant WL/NKL/83/L, granted to A. B-W.

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Figure 1. Expression of Topo2A in pituitary adenomas (optical microscope, magnification 200 x); A: male, 65 yrs: gonadotropinoma, Topo2A index = 0%; B: female, 47 yrs: hormone immunonegative adenoma, Topo2A index = 3.5%
Figure 2. Expression of Topo2A in patients with pituitary adenoma grouped according to their final diagnosis. Median values of Topo2A [IQR] indices are as follows: Acromegaly 0.19% [IQR=0.0-0.84], Cushing disease 1.13% [IQR=0.37-1.21], prolactinoma 0.50% [IQR=0.0-1.01], gonadotropinoma 0.41% [IQR=0.07-1.42], thyrotropinoma 0.0% [IQR=0.0], silent-ACTH 0.95% [IQR=0.89-1.0], hormone immunonegative adenomas 0.8% [IQR=0.65-1.55] (ANOVA test)
Figure 3. Expression of Topo2A in patients in whom no signs of tumor invasiveness were observed and in patients in whom one or more such signs were observed in MRI (n=60) (U Mann Whitney test)
Figure 4. Kaplan-Maier plots of MRI-observed recurrence/progression of pituitary adenoma against Topo2A index in studied patients grouped with respect to values of Topo2A index. A: All patients: Topo2A index > 1% (n=14/20 patients, 70.0% recurrence); Topo2A index ≤ 1% (n=8/40 patients, 20.0% recurrence), B: patients with hormone immunonegative adenomas: Topo2A index > 1% (n=6/8 patients, 75% recurrence); Topo2A index ≤ 1% (n=2/11 patients, 18.2% recurrence)
Figure 5. ROC curve for Topo2A expression as predictor of tumor recurrence/progression, confirmed by MRI.