

**POLYAMINE METABOLISM IN BRAIN TUMORS**

Thesis for degree of Doctor of Philosophy

by  
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## INTRODUCTION

Histopathological classification plays an important role in determining the adequate therapy of intracranial neoplasms. A routine histological processing consists of different staining and immunohistochemical reactions followed by light-microscopical evaluation. The histological diagnosis is based on morphological features, but functional properties such as the expression of oncogenes or the activity of enzymes involved in cell division remains unknown, therefore the chosen therapy can sometimes be proven insufficient. It is important to find any markers of rapidly proliferating neoplastic tissue in order to assess the exact grade of malignancy, and to gain information about the tendency to progression.

### Biological role of polyamines

Polyamines are present in all eukaryotic cells, including cells of the central nervous system. These molecules (spermidine, spermine and their metabolic precursor putrescine) have strong positive charge and relatively small size, thus they can get into interaction with intracellular proteins and nucleic acids. They help the stabilization and condensation of DNA, stimulate translation and increase its' fidelity, and for this reason they play a fundamental role in the regulation of cell differentiation, cellular growth and regeneration. Without polyamines cell division stops in S-phase; the synthesis of DNA decreases and the mitosis is interrupted.

Ornithine decarboxylase (ODC) is the first rate limiting enzymes of polyamine biosynthesis, and one of the most highly regulated eukaryotic enzymes. Increased expression of ODC is a critical factor contributing to oncogenesis. In neoplastic, rapidly proliferating tissue, an elevated activity of ODC has already been described. Many factors induce ODC activity, and therefore cellular polyamine uptake occurs both in normal cells with rapid proliferation and in tumor cells. Various growth stimuli cause highly increased ODC activity, suggesting that the enzyme might be a proto-oncogene product. Many investigation on cellular biology have been performed to understand the highly complicated mechanism of ODC regulation. Despite it has been shown that ODC is regulated at the transcriptional, the translational, and at the post-translational level; the exact mechanism of regulation still remains unknown.

### The role of polyamine-metabolism in tumors

Previous studies of histopathologically different extraneural malignant tumors have shown that polyamine biosynthesis is activated in human neoplasms. A positive correlation between ODC activity and malignancy has already been described in experimental and human gliomas. Thus, polyamines and ODC have been considered to be useful markers for the diagnosis of malignant tumors, and it has been supposed that activation of ODC represents an early expression of malignancy.

### Perspectives in investigation of polyamine metabolism in brain tumors

1. Ependymomas represent 5-6 % of intracranial gliomas; the majority of them benign, but anaplastic forms occur too, resulting in a very bad clinical outcome with an average survival of is 12-20 months. The 3 years mortality rate reaches up to 100 % in many statistics. Since these tumors are radiosensitive, irradiation might influence the outcome. Determining activity of polyamine metabolism in ependymomas could contribute to evaluate the actual grade of malignancy.
2. Astrocytomas are the most frequently observed intraaxial brain tumors. In case of radical removal with postoperative irradiation and chemotherapy in case of anaplastic astrocytomas (AA) one year survival is 60-73 %, whereas in case of glioblastoma multiforme (GBM) it is 35-63 %. The two years survival is only 38-50 % and 8-12 %,

respectively. The progression of astrocytomas has already been described many times, and it is also known, that recurrence of astrocytomas shows frequently higher grade of malignancy. One of the greatest challenges is to evaluate the exact diagnosis in low grade gliomas, and to predict their tendency to progression. Generally, grade II astrocytomas are advised to treat without irradiation, but in cases of recognising possibility to malignization, radical removal with postoperative X-ray therapy might prevent progression.

3. Meningiomas are most frequently diagnosed intracranial tumors of non-glial origin representing 15-20 % of all intracranial tumors. Their histological classification is based on morphological features, but the investigation of these parameters is not always sufficient to safely distinguish the atypical and anaplastic forms, respectively. In spite of their usually benign appearance and their generally total removal, recurrency rate of typical meningiomas is 2-9 %, while that of atypical ones takes 29-50 %. These data indicate the necessity of further investigations. Determining polyamine metabolism in these tumors could produce additional information about their ability to recur, which might be a useful factor in evaluating the exact prognosis.

## OBJECTIVES

1. By evaluating the activity of polyamine metabolism in ependymomas we tried to get information to determine the exact grade of malignancy. We compared grade II ependymomas to other grade II gliomas by estimating the intratumoral concentration of polyamines, and the activity of ODC.
2. In order to get closer to the detection of tendency to progression in astrocytomas, we determined the expression of the ODC gene.
3. In meningiomas we searched correlation between the expression of ODC gene and the rate of recurrency.
4. By measuring the intratumoral quantity of ODC mRNA and the activity of ODC in the same samples, we made an attempt to clarify some aspects of the regulational mechanisms of this enzyme.

## MATERIALS AND METHODS

The surgical specimens were obtained during operation at the Department of Neurosurgery of the University of Cologne from 1991 to 1998 and at the Department of Neurosurgery of the University of Debrecen from 1995 to 1998. Samples from intracranial neoplasms were frozen in liquid nitrogen immediately after excision and stored at  $-80^{\circ}\text{C}$  until analysis.

### Ependymomas

We compared grade II ependymomas (n = 11) to other grade II gliomas (n = 69) and to peritumoral, non-neoplastic tissue (n = 52). The group of other gliomas consisted of astrocytomas, oligodendrogliomas and mixed oligo-astrocytomas.

### Astrocytomas

We investigated 24 samples of astrocytomas, which were arranged by histopathological classification. Four groups were created: astrocytomas grade II (A II: n = 8),

astrocytomas grade III (A III: n = 8), glioblastoma multiforme (GBM: n = 8), and peritumoral samples (n=5) served as reference group.

### Meningiomas

We compared three groups of meningiomas as follows; group 1: meningiomas without recurrence during an 8.4 years average follow-up period (M - R: n = 8); group 2: meningiomas which later recurred (M + R: n = 14; average time to recurrence is 3.0 years) and group 3: recurrent tumors of the second group (R: n = 14).

In case of ependymomas we determined the intratumoral concentration of polyamines putrescine, spermidine and spermin, and the activity of ornithine decarboxylase. In case of astrocytomas and meningiomas we measured the relative intratumoral quantity of ODC mRNA and the activity of ODC; and in meningiomas we determined the Ki-67 index and the number of mitoses.

### Histological processing

For conventional histopathological evaluation 10 µm thick cryostat sections were taken from each sample and stained with hematoxylin and eosin. Tumors were classified according to the criteria of the World Health Organization (WHO) revised in 2000.

For Ki-67 labeling, the sections were air dried for 2 h at room temperature and then fixed in cold acetone for 10 min. After rinsing in Tris buffer, the sections were preincubated with 3 % normal swine serum to prevent background staining. We used a polyclonal Ki-67 antibody (A 0047) diluted at 1 : 10 with 30 min incubation at room temperature. The antibody binding was detected by the APAAP (alkaline phosphatase antialkaline phosphatase) or ABC (streptavidin-peroxidase) procedure with aminoethylcarbasole as chromogen and hematoxylin counterstaining. The labeling index was based on counting of at least 1000 tumor cells in an area of the section with the highest labeling frequency. Vascular, inflammatory and xanthomatous cells were excluded.

### Biochemical analysis

For determination of polyamine concentrations, tumor samples were homogenised with 0.1 mol/l HCl in methanol at -20°C. Tissue homogenates were extracted twice with 0.6 mol/l HClO<sub>4</sub>. After centrifugation the supernatant was neutralised with 3 mol/l KOH. After derivatisation with o-phthalaldehyde polyamines were separated on a reverse phase Partisil 10 ODS 3 high performance liquid chromatography column and quantified by fluorescence detection.

For ODC analysis tissue samples were homogenised at 4°C with 25 vols Tris/HCL-buffer (50 mM, pH 7.2, supplemented with 5 mM dithiothreitol). The test mix was composed of the tissue sample in Tris/DTT buffer, supplemented with 54 µM pyridoxal phosphate and L(1-<sup>14</sup>C)ornithine (specific activity 55 mCi/mmol) in a total volume of 130 µl. After 1 h incubation in sealed tubes at 37°C, ODC activity was determined by measuring the release of <sup>14</sup>CO<sub>2</sub> in a liquid scintillation counter.

MRNA levels for ODC and β-actin were assessed by quantitative competitive RT-PCR (reverse transcriptase polymerase chain reaction). β-actin as a house keeping gene was used as an internal standard. The target to be amplified spanned more than 1 exon with 401 bp for ODC and 440 bp for B-actin, respectively. The mRNA-specific competitors were engineered performing SOE-PCR (splicing by overlap extension PCR) with a single base mutation so that a unique recognition sequence of the restriction endonuclease Bgl II (AGATCT) was inserted into the β-actin wild-type cDNA at 207 bp and a unique recognition sequence of the restriction endonuclease Bam HI (GGATCC) into the standard cDNA of

ODC at 209 bp. Total RNA of tumor samples was isolated and quantified spectrophotometrically at 260 nm. One microgram of total RNA per sample was exposed to 20 U of AMV (avian myeloblastosis virus) reverse transcriptase. The resulting cDNA was combined with five increasing concentrations of the specific competitor. PCR was performed in a 50  $\mu$ l reaction volume containing 10  $\mu$ l of diluted cDNA with the competitor, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.2  $\mu$ M each sense and antisense primer and 0.6 U of DNA-polymerase. Reaction mixtures were heated to 95°C for 3 min followed by 31 cycles at 95°C for 30 sec, 60°C for 40 sec and 72°C for 40 sec with a final extension step for 7 min at 72°C.

Aliquots of the reactions were digested with Bam HI and Bgl II, respectively. Negative and digestion controls were done. The reaction products were loaded on a 2 % agarose gel and separated electrophoretically. The DNA was stained with ethidium bromide, and quantified using high-energy ultra-violet radiation and a computerized image processing system. ODC mRNA was calculated relative to the  $\beta$ -actin mRNA.

Data given are average  $\pm$  standard deviation (SD). In case of gliomas, significance of differences between the groups of grading were analysed using one-tailed Student's T-test. In case of meningiomas, significance between the groups (M - R) and (M + R) was estimated by unpaired Mann-Whitney's *U* test. To compare the group (M + R) with its corresponding recurrences (R), paired Wilcoxon signed-rank test has been used.

## RESULTS

### Ependymomas

We found low *ODC activity* in the peritumoral, non-neoplastic tissue ( $0.9 \pm 0.6$  nmol/g/h) and in grade II gliomas ( $3.3 \pm 4.4$  nmol/g/h). Surprisingly, in grade II ependymomas the ODC activity ( $11.3 \pm 13.8$  nmol/g/h) was significantly higher than in peritumoral samples ( $p \leq 0.0001$ ), and in other, grade II gliomas ( $p \leq 0.001$ ).

In grade II ependymomas the level of *putrescine* ( $139.4 \pm 118.7$  nmol/g) was significantly higher than in the peritumoral tissue ( $47.6 \pm 27.9$  nmol/g,  $p \leq 0.0001$ ), but there was no significant difference between grade II ependymomas and other gliomas ( $122.4 \pm 106.1$  nmol/g).

The *spermidine* level showed no significant differences between the groups examined.

The *spermine* level was significantly higher in grade II ependymomas ( $188,8 \pm 151,7$  nmol/g) than in grade II gliomas ( $77.6 \pm 81.2$  nmol/g,  $p \leq 0.05$ ) and in the peritumoral tissue ( $91.7 \pm 46.1$  nmol/g,  $p \leq 0.01$ ), but there was no significant difference in the other gliomas comparing to the peritumoral tissue.

### Astrocytomas

The *ODC activity* in high grade tumors was significantly higher than in peritumoral brain tissue (peritumoral tissue:  $0.9 \pm 0.3$  nmol/g/h, A III:  $7.9 \pm 4.4$  nmol/g/h,  $p \leq 0.01$ , GBM:  $26.5 \pm 15.7$  nmol/g/h,  $p \leq 0.01$ ). Comparing the ODC activity of grade II astrocytomas ( $2.0 \pm 1.5$  nmol/g/h) to peritumoral tissue, the difference was not significant, but it differed significantly from grade III astrocytomas ( $p \leq 0.05$ ) and from glioblastoma multiforme ( $p \leq 0.001$ ). Interestingly, ODC activity was significantly higher in glioblastoma multiforme, than in grade III astrocytomas ( $p \leq 0.01$ ).

Determining the *quantity of ODC mRNA*, we found significant difference between grade II astrocytomas ( $3.2 \pm 1.5$  mU/U  $\beta$ -aktin) and peritumoral brain tissue ( $1.4 \pm 0.3$  mU/U  $\beta$ -aktin,  $p \leq 0.05$ ). The quantity of ODC mRNA in glioblastoma multiforme was also elevated significantly

( $4.9 \pm 3.6$  mU/U  $\beta$ -aktin,  $p \leq 0.001$ ), comparing to the peritumoral tissue. The value of grade III astrocytomas ( $4.7 \pm 3.7$  mU/U  $\beta$ -aktin) did not differ statistically from the value of other groups.

### Meningiomas

We found no significant difference in *ODC activity* between meningiomas with recurrence (M + R:  $6.9 \pm 6.6$  nmol/g/h), and tumors without recurrence (M – R:  $3.7 \pm 3.8$  nmol/g/h), but ODC activity was significantly higher in recurrent meningiomas (R:  $13.4 \pm 13.7$  nmol/g/h,  $p \leq 0.001$ ), than in their primary tumors.

*Quantity of ODC mRNA* was significantly higher in meningiomas with recurrence (M + R:  $7.1 \pm 5.2$  mU/U  $\beta$ -aktin), than in tumors, which did not recur during the follow-up period (M – R:  $2.1 \pm 1.0$  mU/U  $\beta$ -aktin;  $p \leq 0.001$ ). The quantity of ODC mRNA in recurred meningiomas was significantly lower (R:  $5.5 \pm 4.2$  mU/U  $\beta$ -aktin,  $p \leq 0.01$ ) than in their primary tumors.

The *Ki-67 index* was lowest in meningiomas without recurrence (M - R:  $2.5 \pm 1.7$  %). Meningiomas with later recurrence had higher value (M + R:  $6.8 \pm 6.9$  %). The Ki-67 index was significantly higher in recurred tumors (R:  $8.2 \pm 6.0$  %) than in the first group ( $p \leq 0.001$ ).

The number of mitosis elevated together with the Ki-67 index: it was in meningiomas without recurrence (M – R)  $0.7 \pm 0.5/\text{mm}^2$ , in meningiomas with later recurrence (M + R)  $3.6 \pm 6.4/\text{mm}^2$ , and in their recurrences (R)  $5.1 \pm 9.5/\text{mm}^2$ , but we did not find any significant differences by statistical analysis.

## DISCUSSION

Previous studies on histopathologically different extracranial malignant tumors have shown that polyamine biosynthesis is activated in human neoplasms. A positive correlation between ODC activity and grade of malignancy has already been described in experimental and human gliomas. Thus, polyamines and ODC have been considered to be useful markers for the diagnosis of malignant tumors, and it has been suggested that activation of ODC represents an early expression of malignancy. Many investigations on cellular biology have been performed to understand the highly complicated mechanism of ODC regulation. Although it has been shown that ODC is regulated at the transcriptional, at the translational and at the post-translational level, the exact mechanism of regulation still remained unknown. In previous studies a slightly elevated ODC mRNA level was found in cancerous colonocytes relative to normal colonocytes, suggesting that colonocyte ODC activity is regulated post-transcriptionally.

We searched correlation between the proliferative potential of intracranial tumors and activity of polyamine metabolism. Our investigations aimed to the most frequent intracerebral tumors (astrocytomas), to the most frequent extracerebral but intracranial tumors (meningiomas) and to the ependymomas.

Our findings supported the theory about the elevated activity of polyamine metabolism in proliferating neoplastic tissue. The level of some polyamines (putrescine and spermine) and the activity of ODC proved to be higher in grade II ependymomas than in the non-neoplastic brain tissue. Furthermore, the activity of polyamine metabolism was evidently increased in grade II ependymomas in relation to grade II other gliomas. Ependymomas arise from the ependymal cells, which originate directly from the neural tube. Thus, it may be suggested that high ODC activity and polyamine concentrations in ependymomas reflect the developmental characteristics and the proliferative potential of the ependymal and subependymal cell matrix. We hope, that the results of determination of the polyamine metabolism in ependymomas might help the establishment of postoperative oncotherapy.

We also investigated the polyamine metabolism in astrocytomas by comparing the activity of ODC with the quantity of ODC mRNA. The ODC activity was significantly higher in grade III astrocytomas and in glioblastoma multiforme than in the peritumoral brain tissue, and the ODC activity correlated well with malignancy. The ODC activity in grade II astrocytomas was similar to the value of the peritumoral tissue, but the quantity of ODC mRNA showed a marked elevation in grade II tumors, which calls the attention to the proliferative potential of the low grade astrocytomas. This difference of changes between ODC activity and quantity of ODC mRNA was detected also in grade III astrocytoma and in GBM. The ODC activity was significantly higher in GBM than in grade III tumors, but the quantity of ODC mRNA was similar in both groups. These results suggest the role of post-transcriptional regulatory mechanism of ODC.

ODC mRNA level, enzyme activity, Ki-67 index and number of mitoses were evaluated in meningiomas in order to examine the possible role of ODC gene expression in the recurrent growth of these tumors. We found no direct correlation between ODC activity and Ki-67 index, indicating that both parameters reflect different aspects of tumor proliferation. ODC activity was highest in recurrent tumors without any parallel elevation of mRNA level. This unrelated change could emphasize the predominant role of post-transcriptional regulatory mechanism of ODC in the recurrent meningioma growth. We measured a significant elevation of ODC mRNA level in meningiomas with later recurrence in comparison to meningiomas without recurrence. These findings could underline the possible role of ODC gene expression for recurrence of meningiomas.

## CONCLUSIONS

1. The polyamine metabolism is more increased in ependymomas, than in other gliomas. This suggests a more prominent proliferative potential of ependymomas comparing them to other gliomas of the same grade.
2. Determination of polyamine levels in ependymomas served no evident correlation with the grade of malignancy.
3. We detected significantly high ODC mRNA level in low grade astrocytomas, which underlines the inclination to progression of these tumors.
4. The elevation of ODC activity both in astrocytomas and in meningiomas showed no parallel changes with the quantity of mRNA, which emphasizes the role of post-transcriptional mechanism in regulation of ODC expression.
5. The quantity of ODC mRNA was significantly higher in meningiomas with later recurrence than in meningiomas without recurrence. This might suggest that ODC mRNA level could be a prognostic factor in predicting recurrence of meningiomas.
6. ODC mRNA seems to characterize the potential of neoplastic cell proliferation, while ODC activity represents a marker of the actual grade of malignancy.

## List of scientific presentations

### Publications which the theses are based on:

1. Klekner A, Röhn G, Schillinger G, Schröder R, Klug N, Ernestus RI: ODC mRNA as a prognostic factor for recurrence in meningiomas. J Neuro-oncol. 53: 67-75, 2001 *IF: 1,581*
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1. Klekner A, Ernestus RI, Röhn G, Molnár P, Csécei Gy, Klug N: Polyamine metabolism in ependymomas. Pathol Int, 46, S1: 625, 1996, *IF: 0,83*
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3. Schillinger G, Röhn G, Klekner A, Schröder R, Klug N, Ernestus RI: ODC-elevation in astrocytomas is regulated posttranscriptionally. Zentralblatt für Neuro-chirurgie Suppl, 1: 79, 2000, *IF: 0,939*

**IF of publications which the thesis are based on: 7,135**

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1. Klekner A, Szentirmay Z, Németh T, Szilágyi L, Aradi J, Ádám B, Szatmári I, Virág J, Molnár P: A complex approach for the precise determination of brain tumors' grade. Congress of the Hungarian Society of Pathologists and of the Hungarian Division of the International Academy of Pathology, August 26-29, 1998 Gyula, Hungary
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