

Hiding in the Shadows: *CPOX* Expression and 5-ALA Induced Fluorescence in Human Glioma Cells

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Abstract Protoporphyrin IX (PpIX) is widely used in photodynamic diagnosis. To date, the details of molecular mechanisms underlying PpIX accumulation in malignant cells after 5-ALA administration remain unclear. The fluorescence of PpIX was studied in human glioma cells. Several cell cultures were established from glioma tumor tissue to study the differences between fluorescence-positive and fluorescence-negative human glioma tumors. The cell cultures demonstrated fluorescence profiles similar to those of source tumor tissues, which allows us to use these cultures in experimental research. Dynamics of the rates of synthesis and degradation of fluorescent protoporphyrin IX was studied in the cultures obtained. In addition, the expression of *CPOX*, an enzyme involved in PpIX synthesis, was evaluated. mRNA levels of heme biosynthesis enzymes were analyzed, and PpIX fluorescence proved to correlate with the *CPOX* protein level, whereas no such correlation was observed at the mRNA level. Fluorescence intensity decreased at low levels of the enzyme, which indicates its critical role in PpIX fluorescence. Finally, the fluorescence intensity proved to correlate with the proliferative activity.

Keywords PpIX · 5-ALA · Glioma · Cell cultures · *CPOX*

Introduction

Gliomas are the most common malignant brain tumor associated with rapid progression and poor prognosis. Surgical resection is one of the main approaches to brain glioma therapy. It leads to a temporary remission, the duration of which depends on the completeness of tumor resection. This is complicated by the absence of pronounced boundaries of malignant gliomas. The surgical efficacy (of operations) under white light is quite low since only the visible tumor focus, which is often necrotic, is removed. The proportion of operations with complete tumor removal under white light is at most 20 % of total operations [1]. Photodynamic diagnosis is the most promising approach to increase the efficacy of tumor resection. This method relies on specific accumulation of a photosensitive compound in malignant cells. Protoporphyrin IX (PpIX) is widely used in photodynamic diagnosis. It shows pronounced fluorescence when exposed to light at 635 nm. PpIX is a metabolite of 5-aminolevulinic acid (5-ALA), a nonfluorescent derivative of amino acids. Both PpIX and 5-ALA are intermediate products in heme biosynthesis. Systemic administration of 5-ALA leads to the accumulation of fluorescent PpIX inside malignant cells. This allows precise surgical resections preserving the neighboring normal brain regions. The first reports on 5-ALA application in neurosurgery date back to late 1990s [2, 3]. At the same time, no 5-ALA-induced PpIX fluorescence was observed in a number of cases, particularly, in metastatic tumors of the brain. It was proposed that fluorescence can be observed only in 70 % of tumors after 5-ALA administration [4–6].

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To date, the details of molecular mechanisms underlying PpIX accumulation in malignant cells after 5-ALA administration remain unclear. Experimental data demonstrated that active PpIX accumulation in malignant cells can be due to increased 5-ALA permeability in tumor tissues, active 5-ALA capture by malignant cells, and altered activity of enzymes catalyzing PpIX conversion to heme [7]. PpIX fluorescence was shown to correlate with the proliferation rate of tumor cells, neoangiogenesis (CD31-positive) [8], blood-brain barrier permeability [9], and other factors. In addition, abnormal synthesis of PpIX in the cell can be due to altered activity of enzymes involved in the process. PpIX synthesis from 5-ALA is catalyzed by the following enzymes: ALAD, HMBS, UROS, UROD, CPOX, and PPOX [10]. Normally, PpIX is a heme precursor in the heme biosynthesis pathway. Heme is largely synthesized in the liver and bone marrow; however, this process goes in nearly all cells of the body. Alterations of the activity of the enzymes of this synthetic pathway cause diseases coined as porphyrias. In addition to seven enzymes mentioned above, heme synthesis depends on ALAS, which catalyzes glycine conversion to 5-ALA. However, ALAS is not required for PpIX synthesis from 5-ALA in tumor visualization since 5-ALA is administered rather than synthesized by cells. PpIX synthesis from 5-ALA occurs both in mitochondria and cytosol and is catalyzed by the enzymes mentioned above [11]. ALAD, HMBS, UROS, UROD, CPOX, and PPOX mediate PpIX synthesis, while FECH converts it to nonfluorescent heme. δ -Aminolevulinic acid dehydratase (ALAD) mediates the formation of porphobilinogen from two 5-ALA molecules; hydroxymethylbilane synthase (HMBS) converts four porphobilinogen molecules to a tetrapyrrole compound, hydroxymethylbilane; uroporphyrinogen III synthase (UROS) converts hydroxymethylbilane to uroporphyrinogen III; the latter is decarboxylated by uroporphyrinogen decarboxylase (UROD) to form coproporphyrinogen III; coproporphyrinogen III is transported to the mitochondria where it undergoes oxidative decarboxylation catalyzed by coproporphyrinogen oxidase (CPOX) generating protoporphyrinogen III. Protoporphyrinogen III is further oxidized by protoporphyrinogen oxidase (PPOX) to produce PpIX. At this stage, it can be used in photodynamic diagnosis. At the next step, ferrochelatase (FECH) converts PpIX to nonfluorescent heme [11]. A possible reason for the lack of fluorescence of some tumors could be due to either drop of the enzyme synthesis or alterations of structure and function of the enzymes required for PpIX synthesis. Analysis of correlations between enzyme concentrations and fluorescence intensity in tumor tissues demonstrated that fluorescence intensity can depend on CPOX [12, 13],

as well as FECH [14]. In this research, primary and passaging cell cultures, derived from fluorescence-positive and fluorescence-negative gliomas, have been used. In addition, it has been studied whether all cell within the population are homogenous, that is, fluorescence-positive or fluorescence-negative. Correlation has been found between the CPOX concentration and the capacity to accumulate PpIX. In this research, an expression of CPOX gene has been studied by measuring of mRNA and protein synthesis levels, which revealed new aspects of the lack of fluorescence of tumor cells.

Materials and Methods

Cell Cultures

Surgical human glioma samples were transferred to sterile Hanks solution (PanEko) containing 2 μ g/ml gentamicin. Within 2 h, the samples were reduced in Versene (PanEko) to a size of \sim 1 mm² while necrotic areas were discarded. The samples were treated with 0.25 % trypsin at 37 °C for 30 min and further disaggregated by passing through a 22G needle and a 70 μ m nylon membrane 3–5 times. After washing three times in sterile Hanks solution (800 g for 3 min at 37 °C), the pellet was resuspended in complete DMED/F12 containing 10 % FBS and 2 mM L-glutamine and plated in 25 cm² flasks (Corning). On the next day, the culture medium was replaced with a fresh one. Cultures were passed by replating at 1:1 upon reaching 80–100 % confluence.

Fluorescence Analysis

Cell cultures were incubated with 100- μ M 5-ALA for 2–24 h. Cells were washed twice with an excess of Versene, harvested by 0.25 % trypsin, transferred to a microcentrifuge tube, centrifuged at 800 g for 5 min, and washed twice with PBS. Protoporphyrins were extracted by 50 % isopropanol at 100 μ l per 30-mm culture dish. Fluorescence was analyzed using a Cary Eclipse fluorescence spectrophotometer (Varian) at an excitation wavelength of 405 nm and emission range of 500–700 nm. The dynamics of PpIX anabolism and catabolism was evaluated by normalizing fluorescence intensity to that after 2-h incubation with 5-ALA.

Fluorescence Microscopy

Cells were plated on Cell Imaging Coverglasses (Eppendorf) upon reaching 15–90 % confluence in the medium containing 100 μ M 5-ALA and incubated for 1–24 h. PpIX fluorescence was evaluated after 60, 80, 100, 120, 180, 230, 380 min, and

24 h using an LSM 710 microscope (Carl Zeiss). The excitation wavelength was 488 nm, and PpIX fluorescence spectra were recorded in the range of 470–715 nm.

RT-PCR and qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) and treated with DNase I (Invitrogen) to remove genomic DNA according to the manufacturer's protocol. Reverse transcription was performed using a MMLV RT kit (Evrogen) and random hexamer primers. The obtained complementary DNA (cDNA) was used as the template in RT-PCR and qRT-PCR. The used primers are listed in Table 1. Expression analysis was performed for the following genes: *ALAD*, *HMBS*, *UROS*, *UROD*, *CPOX*, *PPOX*, and *FECH*. QRT-PCR was carried out in a CFX96 thermocycler (BioRad) using a PCR-Komplekt kit in the presence of the SYBR Green dye (Syntol). The PCR program consisted of 45 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. Fluorescence intensity was measured after the elongation stage. A melt curve analysis was conducted to confirm the specificity of PCR products. PCR data were analyzed using the CFX96 software. Hypoxanthine-guanine phosphoribosyltransferase gene (*HPRT1*) was used as the reference gene. Expression was evaluated as the target-to-reference gene levels.

Rapid Amplification of cDNA Ends

3' Rapid amplification of cDNA ends was performed using a FirstChoice RLM-RACE kit (Ambion) according to the manufacturer's instructions. The resulting amplicons were cloned into pGEM-T Easy vector and sequenced.

Southern Blotting

PCR products were transferred to positively charged nylon membranes (Roche) using capillary transfer under denaturing conditions. The probe corresponding to nucleotides 723–1450 of the *CPOX* transcript (NM_000097.5) was labeled using the Biotin DecaLabel DNA Labeling Kit (Fermentas). Hybridization was performed in Church-Gilbert solution.

Western Blotting

Cells were analyzed by Western blotting 48 h after transfection. Monolayer cells were harvested from culture flasks, thoroughly rinsed with an excess of Versene (PanEko) by centrifugation (800 g, 5 min), and the pellet was lysed in the following buffer (100 µl per 10⁶ cells): 60 mM Tris-HCl (pH 6.8), 25 % (v/v) glycerol, 2 % SDS, 5 % (v/v) 2-mercaptoethanol, and 0.01 % (w/v) bromophenol blue. About 40 µg of protein samples were loaded onto a 10 % gel and analyzed by SDS-PAGE. Proteins were transferred

Table 1 Primers used in mRNA quantitation for *ALAD*, *HMBS*, *UROS*, *UROD*, *CPOX*, *PPOX*, and *FECH*. *f* forward primer, *r* reverse primer

Gene	Primer sequences	Annealing temperature (°C)
<i>ALAD</i>	f:GCTACTTCCACCCACTACTTCG r: TCAGGAACATCCGTGACAAAG	60
<i>HMBS</i>	f:AGCTTGCTCGCATACAGACG r: AGCTCCTTGGTAAACAGGCTT	60
<i>UROS</i>	f:GCCAAGTCAGTGTATGTGGTT r: GCAATCCCTTTGTCCTTGAGC	60
<i>UROD</i>	f:TCTCCGACATCCTTGTGTGACC r: CCGTAGGCGTTCTAGGTCCT	60
<i>PPOX</i>	f:TCTGCCGTGGAGTGTGTTGC r: ATGGAACGATGGGTTTGCTCA	60
<i>FECH</i>	f:GGAGATGTTACGACTTCCTTC r: GAATGGTGCCAGCTTATTCTGA	60
<i>HPRT1</i>	f: TGAGGATTTGGAAAGGGTGT r: GAGCACACAGAGGGCTACAA	60
<i>CPOX</i> qRT-primers	f: GCTGGGGTGAGCATTCTGT r: GCATGAGGATTCTTGGGGTGG	60
<i>CPOX</i> RT-PCR primers	f1:CTGCTCCAGGTGATAGTGGGGA f2: GGCGGAGATGTTGCCTAAGAC f3: GCATCAGCTGTGTACTTCAA f4: GATGGCAACAAGCAGTGGT r1: TGGCGTAGAACTCCAGAAT r2: AGAGATCTGGACCATGCTGGT	60

Table 2 Human glioma cell cultures selected to study protoporphyrin IX metabolism

Culture ID	Source tissue	Intraoperative fluorescence status	5-ALA-induced fluorescence status
Kr\fn1	Glioma (diffuse astrocytoma)	Negative	Negative
Ep\fn2	Glioma (oligoastrocytoma)	Negative	Negative
Er\fn3	Glioma (oligoastrocytoma)	Negative	Negative
Mir\fp1	Glioma (glioblastoma)	Positive	Positive
Sus\fp2	Glioma (glioblastoma)	Positive	Positive
Sh\fp3	Glioma (glioblastoma)	Positive	Positive

to a Hybond ECL membrane (Amersham) using a Mini Trans-Blot cell (Bio-Rad) according to the manufacturer's instructions in the buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20 % (v/v) methanol, pH 8.3, at 100 V for 1 h. The membrane was stained with Ponceau Red and thoroughly rinsed with TBS-T buffer. Then the membrane was incubated on a shaker in 5 % defatted milk in TBS-T at room temperature for 30 min and washed three times with TBS-T for 5 min. Proteins were detected using antibodies against CPOX (ab170945, Abcam, 1:4000 dilution). The membrane was incubated with the primary antibodies at 4 °C overnight and washed with TBS-T. Incubation with the secondary peroxidase-conjugated antibodies (1:3000) was carried out at room temperature for 1 h, and the membrane was washed with TBS-T. CPOX detection was performed using an ECL Advance Western Blotting Detection Kit according to the manufacturer's instructions.

LNA Knockdown

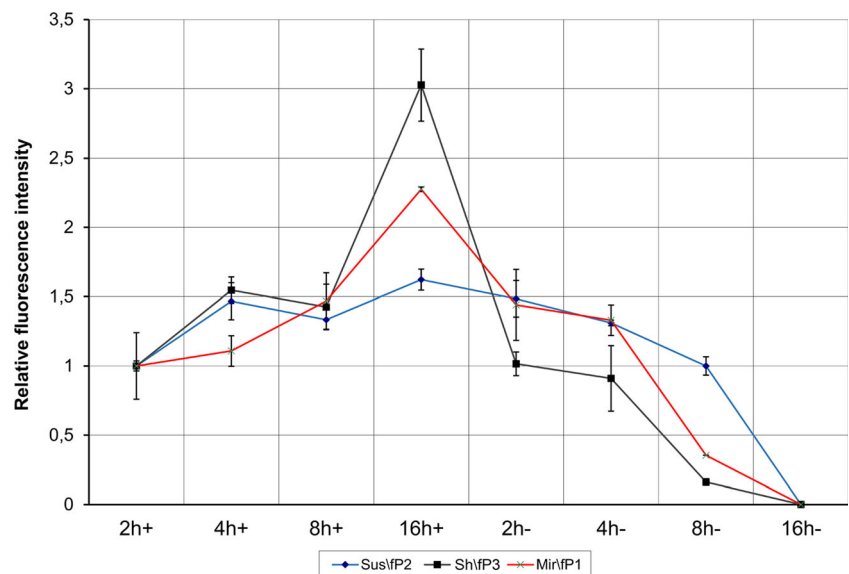
CPOX knockdown was achieved using synthetic phosphorothioated deoxyoligonucleotides with locked nucleic acid (LNA) substitutions at three positions. The following oligonucleotides were used: exonic region, 5'-A*GAGATCTG

G*ACCATGCT*; intronic region, 5'-G*GTTCTCTA*GTTTATATT* (asterisks indicate LNAs). HEK293 cells were transfected with these LNA oligonucleotides (230 nM each) using a TurboFect transfection kit (Thermo Scientific) according to the manufacturer's protocol. The result was confirmed by Western blotting. The effect of decreased CPOX concentration on the level of 5-ALA-induced fluorescence was evaluated using a spectrofluorometer.

Quantitation of Proliferating Cells in Cultures

Cells were plated on 30-mm dishes at 80 % confluence. After 24 h, 0.1 mM BrdU was added to the medium, and 12 h later, cells were fixed with 4 % formaldehyde at 4 °C for 20 min, washed with PBS and distilled water, and treated with 2 N HCl at 37 °C for 20 min. After washing in PBS, cells were exposed to antibodies against BrdU (Abcam, clone IIB5, 1:10) in PBS with Triton X-100 at 4 °C overnight. After washing with PBS, cells were exposed to Cy2-conjugated donkey anti-mouse antibodies at room temperature for 1 h. After washing with PBS, cells were stained with 1 µg/ml bisbenzimidazole H 33342, coated with glycerol, and imaged using an Olympus fluorescence microscope with a digital camera. The number of cells

Fig. 1 Dynamics of synthesis and degradation of fluorescent protoporphyrin IX in fluorescence-positive human glioma cell lines. Abscissa, 2 h⁺, 4 h⁺, 8 h⁺, and 16 h⁺: measurement points 2, 4, 8, and 16 h after 5-ALA administration; 2 h⁻, 4 h⁻, 8 h⁻, and 16 h⁻: measurement points 2, 4, 8, and 16 h after 5-ALA removal following a 16-h incubation with 5-ALA. Ordinate: fluorescence intensity normalized to that after a 2-h incubation with 5-ALA



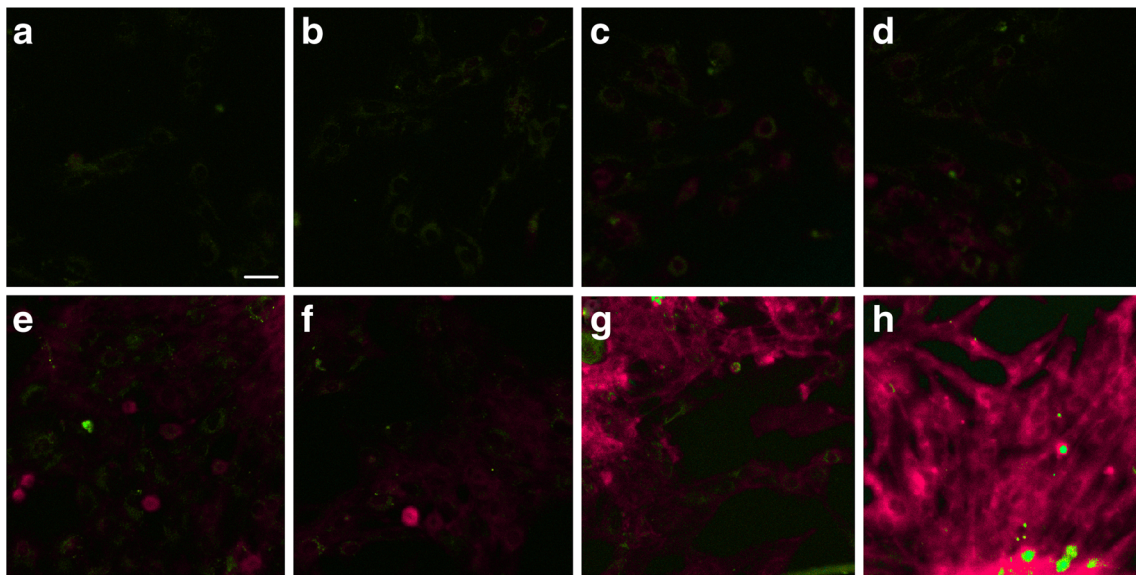


Fig. 2 Fluorescence microscopy of Sh\wp3 cell culture after incubation with 5-ALA. **a–h** Time of incubation with 5-ALA: 60, 80, 100, 120, 180, 230, 380 min, and 24 h. *Red color* indicates 5-ALA-induced fluorescence and *green color* indicates autofluorescence. Certain cells in the vision

field demonstrate low fluorescence; the number of fluorescent cells as well as the overall fluorescence intensity increase with time (**c–h**). *Scale bar* shown on **a** = 50 μ m

positive for bisbenzimidazole and antibodies against BrdU were counted on the images to calculate the proportion of BrdU-positive cells.

Results

All human glioma samples used in this work were obtained during surgery with confirmed diagnosis and intraoperative status of PpIX fluorescence (positive or negative). In total, six human glioma samples were obtained, three had positive

intraoperative PpIX fluorescence status and three had negative one. Cell cultures were established from the tissues, and fluorescence signal was analyzed in them by spectrofluorometry 24 h after culturing with 5-ALA. The fluorescence signal in cell cultures coincided with that during surgery: the cultures obtained from tissues with positive intraoperative fluorescence status demonstrated fluorescence after the incubation with 5-ALA and vice versa. Culture IDs, diagnoses, intraoperative and cell culture fluorescence statuses are given in Table 2.

To describe in detail the capacity of the obtained cell cultures to accumulate PpIX, the dynamics of its accumulation and degradation was evaluated (Fig. 1). Relative changes in the fluorescence intensity were measured after 2, 4, 8, and 16 h of incubation with 5-ALA as well as 2, 4, 8, and 16 h following 5-ALA removal from the medium after a 16-h incubation with 5-ALA. The fluorescence intensity after 2 h of incubation with 5-ALA was taken as normalization point in the three cell cultures. The goal of this experiment was to evaluate the homogeneity of population of the cell cultures according to the fluorescence. The lack of fluorescence of the cells could be due to either conversion of PpIX into non-fluorescent heme or a disturbance of PpIX synthesis. Actually, all studied cell cultures demonstrated different dynamics of PpIX accumulation. In the time interval from 2 to 4 h, Sus\wp2 and Sh\wp3 cell cultures demonstrated a similar increase in fluorescence, which was more intense compared to Mir\wp1 cells. Amazingly, fluorescence intensity decreased in these two cultures in the subsequent time interval from 4 to 8 h of incubation with 5-ALA, while that in Mir\wp1 continued to grow and reached the level of two other cultures after 8 h. In the subsequent 8 h, the rate of PpIX accumulation was

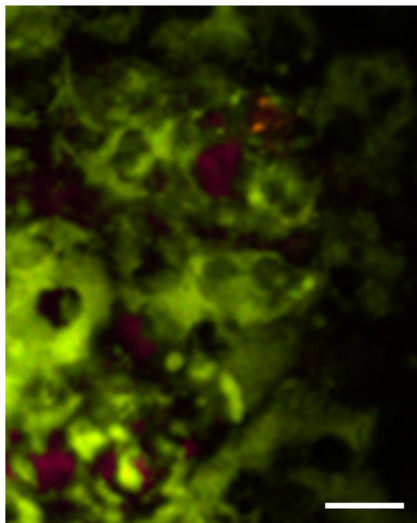
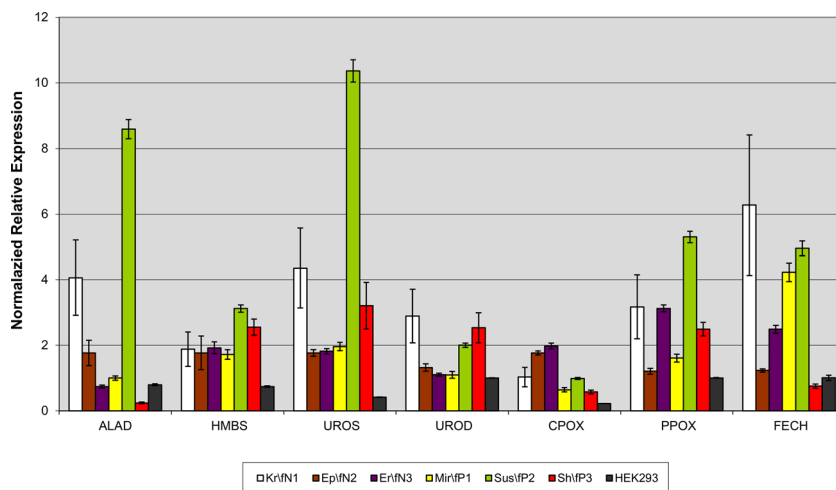


Fig. 3 Fluorescence microscopy of Kr\wp1 cell culture after incubation with 5-ALA. *Red color* indicates 5-ALA-induced fluorescence and *green color* indicates autofluorescence. Isolated fluorescent cells can be observed. *Scale bar* = 50 μ m

Fig. 4 Analysis of mRNA levels of *ALAD*, *HMBS*, *UROS*, *UROD*, *CPOX*, *PPOX*, and *FECH* enzymes involved in heme synthesis in fluorescence-positive (fP) and fluorescence-negative (fN) human glioma cell cultures



different in all three cell cultures: it was most active in Sh\fp3 and least active in Sus\fp2.

The pattern of fluorescence decrease after 5-ALA removal from the medium also differed between all three cell cultures. The period of 0–2 h after 5-ALA removal proved the most heterogeneous: Sh\fp3 demonstrated a dramatic drop in fluorescence intensity, while it was least pronounced in Sus\fp2. The subsequent time intervals of 4–8 and 8–16 h had a relatively similar pattern of active drop in fluorescence intensity, and Sus\fp2 demonstrated a smooth decline in fluorescence intensity throughout these periods. Overall, cell culture-specific patterns of PpIX accumulation resulted from opposite processes of PpIX catabolism and anabolism.

Next, the obtained human glioma cell cultures were tested for PpIX accumulation by fluorescence microscopy. The heterogeneity of PpIX accumulation in cells was analyzed using fluorescence microscopy. Fluorescence-positive cell cultures demonstrated uneven accumulation of PpIX. After an 80-min incubation with 5-ALA, a weak fluorescence signal was observed in only a minor fraction of cells. Fluorescence intensity as well as the number of fluorescent cells increased with incubation time. After 380 min of incubation with 5-ALA, fluorescence was observed in all cells in cultures, and fluorescence intensity increased with incubation time (Fig. 2). Fluorescence-negative cell cultures were analyzed in a similar way: groups of fluorescent

cells could be observed specifically in areas of high cell density; no isolated fluorescent cells were observed in fluorescence-negative glioma cell cultures. The proportion of fluorescent cells in fluorescence-negative cell cultures did not exceed 1 %, which is clearly below the fluorometer sensitivity (Fig. 3).

The expression of enzymes involved in heme synthesis was evaluated by qRT-PCR. We failed to establish a correlation between mRNA levels of most enzymes studied and the fluorescence status of cell cultures. Moreover, the expression levels of the studied genes varied significantly in all studied cell cultures (Fig. 4). At the same time, the level of CPOX mRNA was higher in fluorescence-negative glioma cell cultures than in fluorescence-positive ones ($p < 0.5$).

Analysis of CPOX translation by Western blotting of fluorescence-positive and fluorescence-negative human glioma cultures has demonstrated a correlation between the relative quantity of CPOX and fluorescence status (Fig. 5). The normalized level of the protein was higher in fluorescence-positive cell cultures than in fluorescence-negative ones, which is at variance with the data on a higher level of CPOX mRNA in fluorescence-negative cell cultures.

The discrepancy between the protein and mRNA levels of CPOX could be attributed to different splice variants of CPOX. To test this proposal, we tried to identify and analyze CPOX transcripts. First, oligonucleotide primers were designed to amplify the open reading frame of the expected transcript (primers f1 and r3, see Fig. 6) based on the available CPOX transcript NM_000097.5. However, no PCR product of the expected length has been observed. Initially, we attributed it to a GC-rich region at the 5' end of the studied transcript. However, the expected PCR product was observed when thus shifted reverse primer was used (primers f1 and r2). Similarly, the desired product was observed when a forward primer complementary to the central region of exon 1 (f2) was used together with reverse primer r3. Thus, the failure to amplify the ORF using primers f1 and r3 could be explained by the absence or dramatically small amounts of the full-

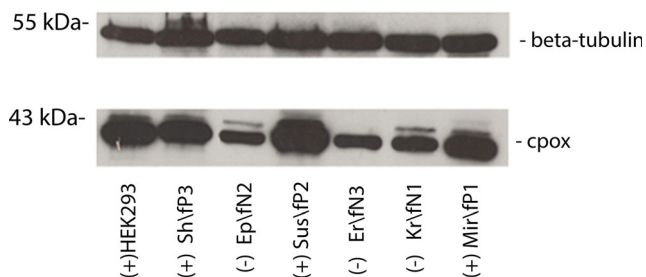
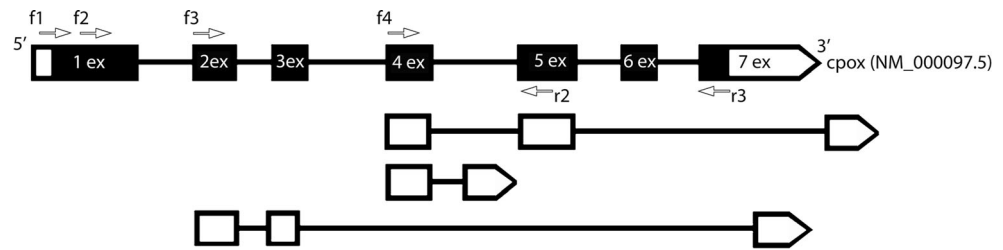


Fig. 5 Western blotting of fluorescence-positive (+) and fluorescence-negative (-) human glioma cultures and Hek293 cells using antibodies against CPOX normalized with antibodies against β -tubulin

Fig. 6 Schematic diagram of primers used and alternative 3'-ends of splice variants identified by 3'-RACE



length *CPOX* transcript. Next, we tried to identify alternative splice variants of the gene. Series of 3' Rapid amplification of cDNA ends has revealed a variation in the 3' ends of *CPOX* transcripts (primers f2, f3 and f3, f4) (Fig. 6).

The obtained PCR amplification products were further analyzed by Southern blotting (Fig. 7). A variety of different transcripts not necessarily coding for the functional *CPOX* has been revealed. Taken together, these data strongly suggest a high variability of *CPOX* splice variants; moreover, the amount of the transcript, mRNA, which is translated into the functional enzyme, could be much less than the total amount the all spliced variants of the transcripts. Consequently, this can explain the discrepancy in the levels of *CPOX* mRNA and protein.

The *CPOX* effect on 5-ALA-induced fluorescence was studied by *CPOX* knockdown in HEK293 cells. Analysis of 5-ALA-induced fluorescence as well as of the enzyme level demonstrated high fluorescence and *CPOX* level in this cell line similar to that in fluorescence-positive glioma cell cultures (Fig. 5). These data demonstrate a similar pattern of *CPOX* functioning in HEK293 cells and studied fluorescence-positive glioma cell cultures. Knockdown was achieved using oligonucleotides with LNA substitutions; the efficiency of this approach has been demonstrated in the recent years [15–17]. Oligonucleotides complementary to an exonic region and an intronic region were selected (see [Materials and Methods](#)).

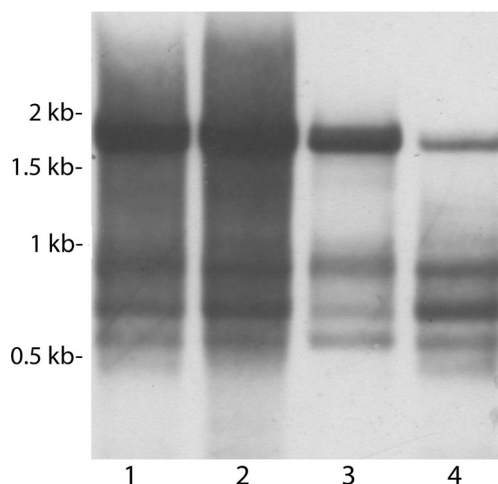


Fig. 7 Southern blotting of 3' rapid amplification of cDNA ends. 1 Mir\fp1, 2 Sus\fp2, 3 Kr\fn1, 4 Ep\fn2. Additional shorter PCR products (500–1500 bp) can be observed

Western blotting analysis demonstrated a roughly twofold decrease in *CPOX* level (Fig. 8), and the decrease in fluorescence intensity in transfected cells was comparable to the decrease in *CPOX* level (Fig. 9). The comparable decrease in the enzyme level and fluorescence intensity indicates a critical role of *CPOX* in PpIX accumulation.

In addition, we have observed a direct and consistent correlation between the rate of cell proliferation and fluorescence status. Human glioma cell cultures with low proliferative capacity demonstrated low PpIX accumulation and were fluorescence-negative. This correlation was further tested in proliferative activity experiments with BrdU. Fluorescence status proved to correlate with the proliferative activity (and hence with the *CPOX* level). Cell cultures demonstrating no protoporphyrin fluorescence had a much lower proliferative activity than fluorescence-positive cell cultures (Fig. 10). This difference was significant. After 12-h incubation, the proportion of BrdU-positive cells was around 4 % in fluorescence-negative cell cultures, while it was 53 % in fluorescence-positive ones.

Discussion

Photodynamic diagnosis widely used in surgical therapy of gliomas relies on the administration of 5-ALA, a natural precursor of fluorescent PpIX. However, no such fluorescence is observed in a number of tumors. In order to explore this phenomenon, we established primary cell cultures from human gliomas. Fluorescence analysis allowed us to divide these cultures into fluorescence-positive and fluorescence-negative groups. Moreover, the established cultures replicated the fluorescence status of the source

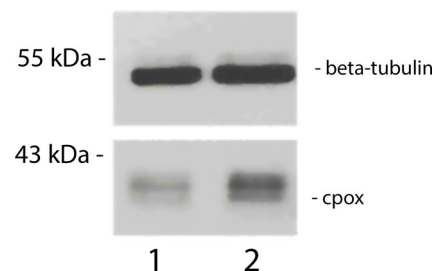
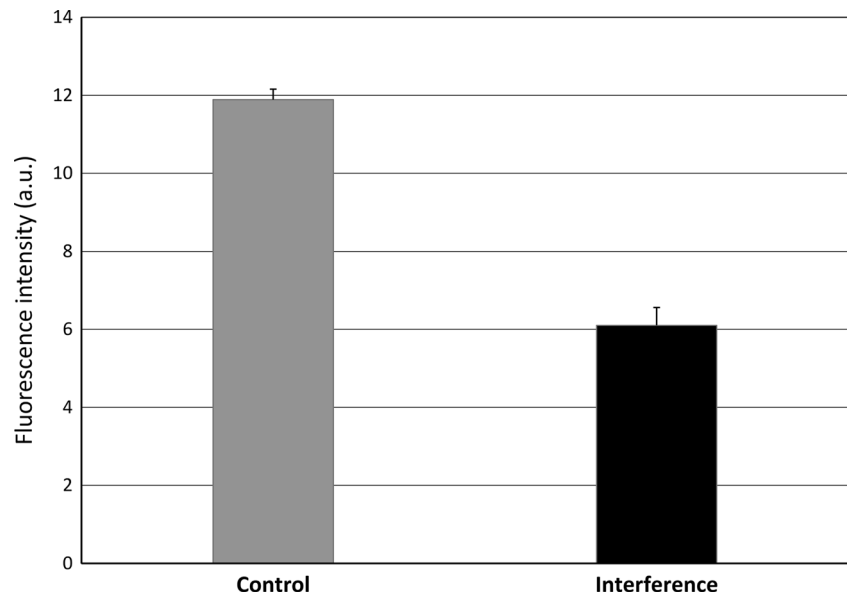


Fig. 8 Knockdown of *CPOX* in HEK293 cells using oligonucleotides with LNA substitutions. Western blotting of lysed HEK293 cells using antibodies against *CPOX*: 1 after transfection with LNA oligonucleotides (equimolar, 230 nM in total), 2 no transfection

Fig. 9 Knockdown of *CPOX* in HEK293 cells using oligonucleotides with LNA substitutions. Spectrofluorometry analysis of lysed HEK293 cells: *Control*, no transfection; *Interference*, after transfection with LNA oligonucleotides (equimolar, 230 nM in total)



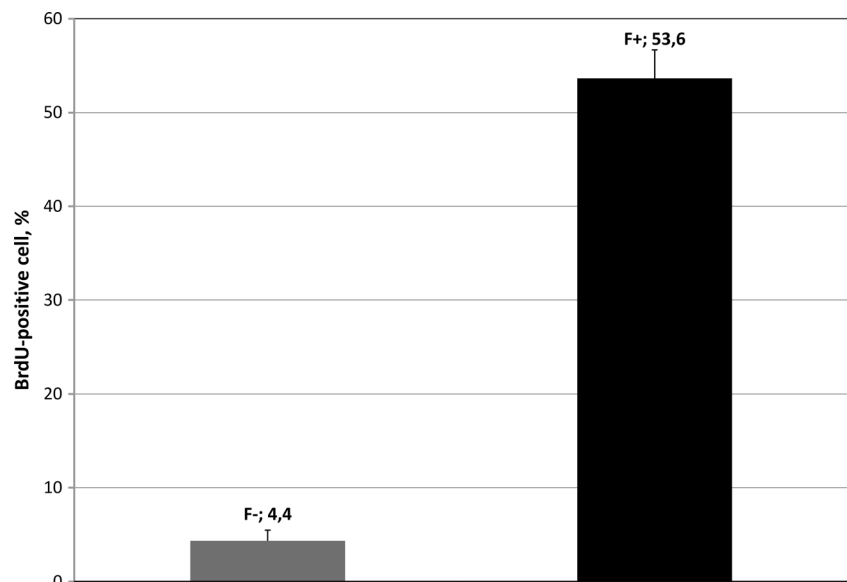
tissues. This made it possible to use the obtained cell cultures as a model system to study PpIX metabolism.

Fluorescence spectroscopy analysis of PpIX accumulation after the incubation with 5-ALA has revealed two features of the studied cell cultures (Fig. 1): the rate of PpIX accumulation varied among fluorescence-positive cell cultures; moreover, fluorescence intensity decreased in the period from 4 to 8 h in two out of three fluorescence-positive cell cultures (no fluorescence signal was observed in fluorescence-negative cell cultures after a 24-h incubation with 5-ALA). One can propose that this decrease in PpIX fluorescence intensity is due to the regulatory processes triggered by excessive PpIX. For instance, heme is a corepressor of ALAD [18] while peripheral-type benzodiazepine receptor (PBR) can bind PpIX and, thus, participate in the regulation of heme synthesis in murine cells

[19]. Overall, this indicates the presence of mechanisms of heme biosynthesis regulation that could underlie the phenomena mentioned above.

Fluorescence microscopy demonstrated different rates of PpIX accumulation among cells in fluorescence-positive cell cultures (Fig. 2). Amazingly, we found groups of adjacent fluorescent cells in fluorescence-negative cell cultures. Such fluorescence was observed only within cell aggregates but not in isolated cells. Roughly, the proportion of fluorescent cells does not exceed 1 %. Apparently, cell fluorescence specifically within aggregates is due to local changes in cell properties; cell density, cell cycle, and pH are known to modulate PpIX accumulation [20]. Such factors can be responsible for the fluorescence observed here. Clearly, PpIX accumulation is a regulated process that can be initiated under specific conditions and can

Fig. 10 Proportion of BrdU-positive cells in fluorescence-positive (F+) and fluorescence-negative (F-) human glioma cell cultures. On average, the difference in proliferation rate was more than ten times (4.4 % in fluorescence-negative vs. 53.6 % in fluorescence-positive)



depend on a specific cell state. The observed heterogeneity in the rate of PpIX accumulation within cell cultures agrees with the general pattern of malignant cells, specifically, their high heterogeneity. Notably, such heterogeneity can be realized at both genetic and epigenetic levels [21–23].

Based on our findings described above and published data [12, 24], on the possible molecular background of different capacity of cell cultures to accumulate PpIX, we selected coproporphyrinogen oxidase (CPOX) for further experiments. Its mRNA and protein levels were evaluated in fluorescence-positive and fluorescence-negative cell cultures. No correlation has been established at the mRNA level, while the relative level of the enzyme was higher in fluorescence-positive cell cultures. This resulted from alternative splice variants of the gene, which is typical for malignant cells [25, 26]. Our analysis not only revealed the presence of such variants but also demonstrated that the total quantity of alternative transcripts is comparable or higher than that of the full-length transcript. Thus, quantitation of CPOX mRNA coding for the functional enzyme is not straightforward and can cause misinterpretation. Our failure to generate a PCR product of the desired length using primers flanking the open reading frame redirects us to the study by Susa et al. discussing two alternative *CPOX* transcripts [27]. It is conceivable that both these transcripts were present in our cell cultures, and the long form level was extremely low. Overall, the revealed transcript variability correlates with the cell culture-specific rate of PpIX accumulation in cells. Different rates of PpIX accumulation can be due to different CPOX level in cells, which consequently depends on the level of the transcript encoding the functional enzyme.

The effect of CPOX level on 5-ALA-induced fluorescence was studied by CPOX knockdown in HEK293 cells. This cell line was also tested for the rate of PpIX accumulation and CPOX level. HEK293 profile proved similar to that of fluorescence-positive cell cultures, which confirms that CPOX functioning is essentially similar in this line and fluorescence-positive cultures and substantiates HEK293 applicability for the analysis. The decrease in fluorescence intensity in proportion with the enzyme level indicates that the reaction catalyzed by CPOX is the bottleneck of heme biosynthesis. Together with the increased level of the enzyme in fluorescence-positive cell cultures, this indicates the critical role of CPOX in PpIX accumulation.

Analysis of cell proliferation rate using the BrdU assay has demonstrated a correlation between 5-ALA-induced fluorescence status and proliferation rate. Overall, cell cultures accumulating PpIX demonstrate a higher proliferative capacity than fluorescence-negative ones. At the same time, analysis of cell aggregates showing fluorescence signal in fluorescence-negative cultures has revealed no increase in the rate of BrdU-positive cells.

Conclusions

This research has shown that PpIX fluorescence intensity in response to 5-ALA administration correlates with the CPOX level, though the correlation with the level of the gene expression is rather nontrivial one. In the fluorescence-negative culture cells of the human glioma, the level of transcription of the CPOX gene is high enough, but the splicing is defected. The level of correct mRNA is low; therefore, the CPOX protein translation is not enough to support PpIX level providing the fluorescence. Moreover, fluorescence intensity decreased at low levels of the enzyme, which indicates its critical role in PpIX fluorescence. Finally, the fluorescence intensity proved to correlate with the proliferative activity; moreover, the cell cultures were heterogeneous regarding fluorescence intensity.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Standard This study was approved by the Ethics Committee of Burdenko Neurosurgical Institute, Russian Academy of Medical Sciences. It was carried out in strict accordance with the ethical principles and scientific standards of the Ethics Committee.

References

1. Barker FG 2nd, Prados MD, Chang SM, Gutin PH, Lamborn KR, Larson DA, Malec MK, McDermott MW, et al. (1996) Radiation response and survival time in patients with glioblastoma multiforme. *J Neurosurg* 84(3):442–448
2. Stummer W, Stocker S, Wagner S, Stepp H, Fritsch C, Goetz C, Goetz AE, Kiefmann R, et al. (1998) Intraoperative detection of malignant gliomas by 5-aminolevulinic acid-induced porphyrin fluorescence. *Neurosurgery* 42(3):518–526
3. Stummer W, Novotny A, Stepp H, Goetz C, Bise K, Reulen HJ (2000) Fluorescence-guided resection of glioblastoma multiforme by using 5-aminolevulinic acid-induced porphyrins: a prospective study in 52 consecutive patients. *J Neurosurg* 93(6):1003–1013. doi:10.3171/jns.2000.93.6.1003
4. Miyatake S, Kajimoto Y, Kuroiwa T (2009) Intraoperative photodynamic diagnosis of brain tumors. *Brain Nerve* 61(7):835–842
5. Savelyeva TA, Loshchenov VB, Ryabova AV, Loshchenov MV, Grachev PV, Isaev MA, Pominova DV, Goryainov SA, et al. (2014) The technology of optical intraoperative diagnosis in neurooncology based on spectroscopic analysis of the nerve tissue. In: Ugryumov MV (ed) *Neurodegenerative diseases: from the genome to the whole organism*. Science World, Moscow, pp. 700–721
6. Wang C, Chen X, Wu J, Liu H, Ji Z, Shi H, Gao C, Han D, et al. (2013) Low-dose arsenic trioxide enhances 5-aminolevulinic acid-

- induced PpIX accumulation and efficacy of photodynamic therapy in human glioma. *J Photochem Photobiol B* 127:61–67
7. Hefti M, von Campe G, Moschopoulos M, Siegner A, Looser H, Landolt H (2008) -aminolevulinic acid induced protoporphyrin IX fluorescence in high-grade glioma surgery: a one-year experience at a single institution. *Swiss Med Wkly* 138(11–12):180–185
 8. Stummer W, Reulen HJ, Novotny A, Stepp H, Tonn JC (2003) Fluorescence-guided resections of malignant gliomas—an overview. *Acta Neurochir Suppl* 88:9–12
 9. Ennis SR, Novotny A, Xiang J, Shakui P, Masada T, Stummer W, Smith DE, Keep RF, et al. (2003) Transport of 5-aminolevulinic acid between blood and brain. *Brain Res* 959(2):226–234
 10. Peng Q, Warloe T, Berg K, Moan J, Kongshaug M, Giercksky KE, Nesland JM (1997) 5-Aminolevulinic acid-based photodynamic therapy. Clinical research and future challenges. *Cancer* 79(12):2282–2308
 11. Ponka P (1999) Cell biology of heme. *Am J Med Sci* 318:241–256. doi:10.1097/00000441-199910000-00004
 12. Taketani S, Furukawa T, Furuyama K (2001) Expression of coproporphyrinogen oxidase and synthesis of hemoglobin in human erythroleukemia K562 cells. *Eur J Biochem* 268(6):1705–1711
 13. Yang DF, Chen JH, Chiang CP, Huang Z, Lee JW, Liu CJ, Chang JL, Hsu YC, et al. (2014) Improve efficacy of topical ALA-PDT by calcipotriol through up-regulation of coproporphyrinogen oxidase. *Photodiagn Photodyn Ther* 11(3):331–341
 14. Hefti M, Hostenstein F, Albert I, Looser H, Luginbuehl V (2011) Susceptibility to 5-aminolevulinic acid based photodynamic therapy in WHO I meningioma cells corresponds to ferrochelatase activity. *Photochem Photobiol* 87(1):235–241. doi:10.1111/j.1751-1097.2010.00821.x
 15. Karkare S, Bhatnagar D (2006) Promising nucleic acid analogs and mimics: characteristic features and applications of PNA, LNA, and morpholino. *Appl Microbiol Biotechnol* 71(5):575–586. doi:10.1007/s00253-006-0434-2
 16. Fluiter K, Mook OR, Vreijling J, Langkjaer N, Højland T, Wengel J, Baas F (2009) Filling the gap in LNA antisense oligo gapmers: the effects of unlocked nucleic acid (UNA) and 4'-C-hydroxymethyl-DNA modifications on RNase H recruitment and efficacy of an LNA gapmer. *Mol BioSyst* 5(8):838–843. doi:10.1039/b903922h
 17. Soifer HS, Koch T, Lai J, Hansen B, Hoeg A, Oerum H, Stein CA (2012) Silencing of gene expression by gymnotic delivery of antisense oligonucleotides. *Methods Mol Biol* 815:333–346. doi:10.1007/978-1-61779-424-7_25
 18. Gibson SL, Havens JJ, Metz L, Hilf R (2001) Is delta-aminolevulinic acid dehydratase rate limiting in heme biosynthesis following exposure of cells to delta-aminolevulinic acid? *Photochem Photobiol* 73(3):312–317
 19. Wendler G, Lindemann P, Lacapère JJ, Papadopoulos V (2003) Protoporphyrin IX binding and transport by recombinant mouse PBR. *Biochem Biophys Res Commun* 311(4):847–852
 20. Moan J, Bech O, Gaullier JM, Stokke T, Steen HB, Ma LW, Berg K (1998) Protoporphyrin IX accumulation in cells treated with 5-aminolevulinic acid: dependence on cell density, cell size and cell cycle. *Int J Cancer* 75(1):134–139
 21. Marusyk A, Polyak K (2013) Cancer. Cancer cell phenotypes, in fifty shades of grey. *Science* 339(6119):528–529. doi:10.1126/science.1234415
 22. Allison KH, Sledge GW (2014) Heterogeneity and cancer. *Oncology (Williston Park)* 28(9):772–778
 23. Navin N, Krasnitz A, Rodgers L, Cook K, Meth J, Kendall J, Riggs M, Eberling Y, et al. (2010) Inferring tumor progression from genomic heterogeneity. *Genome Res* 20(1):68–80. doi:10.1101/gr.099622.109
 24. Chen X, Wang C, Teng L, Liu Y, Chen X, Yang G, Wang L, Liu H, et al. (2014) Calcitriol enhances 5-aminolevulinic acid-induced fluorescence and the effect of photodynamic therapy in human glioma. *Acta Oncol* 53(3):405–413. doi:10.3109/0284186X.2013.819993
 25. Barrie ES, Smith RM, Sanford JC, Sadee W (2012) mRNA transcript diversity creates new opportunities for pharmacological intervention. *Mol Pharmacol* 81(5):620–630. doi:10.1124/mol.111.076604
 26. Wang ET, Sandberg R, Luo S, Khrebtkova I, Zhang L, Mayr C, Kingsmore SF, Schroth GP, et al. (2008) Alternative isoform regulation in human tissue transcriptomes. *Nature* 456(7221):470–476. doi:10.1038/nature07509
 27. Susa S, Daimon M, Ono H, Li S, Yoshida T, Kato T (2003) The long, but not the short, presequence of human coproporphyrinogen oxidase is essential for its import and sorting to mitochondria. *Tohoku J Exp Med* 200(1):39–45