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Thyroid hormone receptors and selected interacting proteins in glial tumors: The analysis of the expression and regulatory potential

PhD thesis– short report

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CONTENTS

Abbreviations

aa – amino acids; cDNA – complementary deoxyribonucleic acid; G – gliosis; G I, GII,GIII or GIV – astrocytoma grade I, II, III or IV; GBM – glioblastoma multiforme; HAT – histone acetyltransferase; HGA – high grade astrocytoma; HGG – high grade glioma; HDAC3 – histone deacetylase 3; kD – kilo Daltons; IAP – The Inhibitors of Apoptosis Protein; LBD – Ligand binding domain; LGA – low grade astrocytoma; NHR – nuclear hormone receptor, NR – nuclear receptor; NCoR – nuclear receptor corepressor; PCR – polymerase chain reaction; Pol II – RNA polymerase II; Q-PCR – quantitative polymerase chain reaction; RNAi – RNA interference; RXR – retinoid X receptor; RT – reverse transcription; SKIP – Ski-interacting protein; SMRT – silencing mediator of retinoid and thyroid hormone receptor; TF – transcription factor; TFIIB – general transcription factor IIB; TR – thyroid hormone receptor; TRE – thyroid responsive element; TSA – trichostatin A;WB – Western blotting; WHO – The World Health Organization

1 SYNOPSIS

Thyroid hormone plays a crucial role in brain development. Contrary to that, a significantly milder phenotype is caused by elimination of both thyroid receptor genes, the TRα and TRβ in knock-out animals, indicating that unliganded receptors severely affect the brain development.

The work described in this thesis was part of a scientific endeavour aimed at the elucidation of the hypothetical possibility that the pathologic involvement of thyroid receptors may be an important part of tumorigenesis of human astrocytic tumors. We investigated the expression of thyroid hormone receptors and their natural dimerization partners, the RXRs, in a collection of bioptic samples. We assayed the level of receptor expression by quantitative PCR, Western blots and by immunocytochemistry in non-malignant gliosis samples, in low grade and high grade gliomas. Special care was given to conduct all examinations in a way that complies with very stringent ethical rules and in all instances, supported and enlarged, and not restricted, the diagnostic process. Samples used for the molecular biology methods were obtained from frozen sections prepared by cryocut apparatus and allowed histopathological control any time during the procedure. The samples were also, because of this, very limited and this did not allow in some cases, to complete all the sets of experiments.

Thyroid hormone receptors` cDNAs of α1, α2, β1 and β2 of expected sizes were efficiently amplified from all examined cases. Quantitative PCR showed a big diversity in the expression of thyroid receptors in all groups of examined samples. Contrary to control experiments performed on total RNA extracted from cell lines, including the glioblastoma cell line U373, which allowed clear quantification, many tumor samples showed disturbed melting curves indicating that no single fragment of mRNA was reverse transcribed and amplified.

Such results were not included in the comparison of the expression of studied transcripts. Analysis of quantifiable experiments indicated that TRα1, TR α2, TR β1 and TR β2 were expressed in the studied samples. A trend, although not statistically significant assuming the null hypothesis, of increased expression of TRα2 and decreased expression of TR β1 was observed. On the protein level, expression of thyroid receptors was studied using commercial antibodies as well as by antibodies obtained from cooperating laboratories. Western blot analysis identified proteins of expected size in case of TRβ1 and TRα2 (TRβ2 was not assayed). The trend of protein expression was similar as that found by Q-PCR, the hallmark was big diversity of the expression levels, the increase of TRα2 and decrease of TRβ1. Antibodies directed against TRα1 detected multiple proteins. On the cellular level, a strong and characteristic expression pattern was obtained in case of TRα2. It was found predominantly in the cytoplasm, although nuclear presence was also detected. The pattern did not differ between non-malignant samples and tumors, but was dramatically stronger in tumors. Strong intranuclear staining was obtained for TRα1 in tumors. The results were compatible with the possibility of formation of multiple protein isoforms of TRα1, but can not be taken as a support of such situation since unspecific interactions could not be excluded. We started experiments aimed at direct mass spectroscopic characterization of proteins recognized by antibodies.

RXRα, RXRβ and RXRγ, dimerizing partners of thyroid receptors, were found present and rather increased in tumors compared to non-tumorous tissues on mRNA level. The increase of RXRγ in tumors was statistically significant ($P<0.05$).

A similar expression pattern was found in the U373 glioblastoma cell line. Levels of detected TRα1, TRα2, TRβ1, TRβ2, RXRα, RXRβ and RXRγ were similar as the highest values observed in malignant astrocytoma. Transfection experiments showed that thyroid receptors in U373 cells are capable of activation of transcription from thyroid responsive promoters but exogenous receptors are necessary for efficient activation of exogenous thyroid responsive promoters. Contrary to that, RXRs seem to be present in sufficient numbers of copies for efficient transcription activation from endogenous responsive promoters.

Next we searched for the expression pattern of cofactors that have hypothetical potential to modulate the function of thyroid receptors. We focused on SKIP, an evolutionarily conserved cofactor shown to interact with several NHRs, Survivin, that was shown to be functionally linked with SKIP and thyroid receptor mediated gene expression and HDAC3,

which is implicated in TR dependent transcription silencing. Interestingly, SKIP was found to be elevated in glial tumors and to follow the elevated expression of Survivin, similarly as it is in *C. elegans*, where, the homologoue of Survivin, *bir-1* is organized with *SKIP* in an operon.

The analysis of the expression of HDAC3 showed markedly elevated and deregulated expression in glial tumors on the level of mRNA, protein and on cellular level. While the distribution of HDAC3 was both nuclear as well as cytoplasmic and moderate in intensity in non-malignant tissues and low grade gliomas, high grade tumors expressed HDAC3 in a focally deregulated pattern that included strongly pronounced cytoplasmic localization. Confocal microscopy and additional colocalization analysis detected nuclear HDAC3 in all tumors examined. We conclude that HDAC3 expression is elevated in human astrocytic tumors and its expression pattern is deregulated at the cellular level in high grade gliomas.

In an attempt to visualize the transcription regulation function of Survivin, which is critically influencing the cell cycle, that in turn projects to transcription regulation, we turned to the heterologous system, *C. elegans*. Here we searched for genes whose expression is affected by BIR-1 loss of function using whole genome microarray experiments in synchronized L1/L2 larvae. *C. elegans* larvae have at the end of the L1 stage most cells postmitotic. The only somatic cell type that is dividing during larval transitions are the seam cells, specialized epidermal cells that keep blast cell (stem cell) character. First we worked out conditions that did not affect cell divisions of seam cells and we used these conditions on large scale larval cultures inhibited for bir-1 by RNA interference. Extracted total RNA was used for whole genome microarrays (Affymetrix). The microarray experiment was done at Microarray facility, NIDDK, NIH. Microarrays identified several collagen and ribosomal genes as candidate targets of *bir-1* inhibition in L1/L2 larval stage. The decreased expression of selected collagen genes in *bir-1* inhibited larvae was confirmed by quantitative RT-PCR. Next, we generated transgenic lines expressing *bir-1* mRNA under a heat shock regulated promoter and searched if *bir-1* overexpression has potential to augment the expression of genes that showed decreased expression in worms treated by *bir-1* RNAi. Overexpression of *bir-1* resulted in a pronounced increase (2 to 5 times) of the expression of these genes. Our findings support the concept that BIR-1, a protein generally regarded as a mitotic factor, is involved in the regulation of transcription during normal development of *C. elegans* and has a strong potency to affect transcription of developmentally active genes if overexpressed.

In conclusion, these analyses indicate that thyroid hormone receptors are likely to be important active players in the regulation of transcription in astrocytic tumors. Our findings argue for often elevated expression of all forms of thyroid receptors. Although TRβ1 shows

tendency to be downregulated in a majority of astrocytomas, in some cases it is strongly upregulated. We hypothetisize that such deregulated expression of thyroid receptors may be explained by incorporation of thyroid receptors in multiple pathways and mechanisms that may be further critically regulated by interaction of thyroid receptors with critical interacting proteins. Indeed, our search for the expression of selected proteins that may critically diverge the function of thyroid receptors found elevated and deregulated HDAC3, SKIP and Survivin.

2 HYPOTHESIS AND AIMS

In addition to specific mutations which cause loss of function of tumor suppressor genes and gain of function of cancer promoting genes, cancer biology depends on cancer supporting transcription profile. Thyroid and retinoid receptors are major differentiation regulatory transcription factors involved in regulation of differentiation. Since thyroid hormone is a critical factor for brain development, we decided to study the expression and function of thyroid receptors in glial tumors. The aim of this project was to characterize expression pattern of thyroid hormone receptors and selected putative cofactors in glial tumors and characterize their function.

3 EXPERIMENTAL DESIGN AND DEVELOPMENT OF THE PROJECT

Focused attempt to characterize the expression of TRs and RXRs in human glial tumors was carried out.

I developed cooperation with the Department of Neurosurgery of the 1st Faculty of Medicine, Charles University and Central Military Hospital in Prague and established a routine for collection of samples obtained during intracranial operations. I was personally present at most operations, participated on collecting samples in a way that was ensuring that only material which would not be used for any other examination was donnated for this study. The informed consent of the use of the bioptic material for scientific purposes was obtained from each patient prior to the study in accordance and with the approval of the Ethics Committee of the Central Military Hospital, Strešovice, Prague and the Ethics Committee of the 1st Faculty of Medicine, Charles University and Faculty Hospital.

We identified the presence of TRs/RXRs in diffusely infiltrating astrocytomas and benign gliosis. We observed that the expression pattern was strikingly deregulated, with

varying levels of expression and in some tumors considerably elevated. There was indication of a tendency of elevated levels of TRα2, TRα1, RXRα and RXRγ while TRβ1 was slightly decreased.

This led us to conclussion that thyroid receptors and RXRs, are likely to be part of cancer biology, as modified proteins or based on interaction with proteins which have potential to diverge their function.

In search for such proteins, we have focused on SKIP, the evolutionarily conserved cofactor of nuclear receptors, Survivin, which is functionally linked to SKIP in *C. elegans* (Kostrouchova et al., 2002; Kostrouchova et al., 2003b) and HDAC3, which is known to be able to bind to thyroid receptors in absence of the ligand through the interaction with Cyclin D1 and inhibit thyroid hormone dependent transcription (Lin et al., 2002). HDAC3 expression was significantly elevated in malignant tumors and we decided to study its expression in detail. The study confirmed that HDAC3 is elevated and deregulated on cellular level in malignant astrocytomas.

Survivin and SKIP were found deregulated and elevated as well. We decided to prolongate a study earlier done in our laboratory that showed that BIR-1, the *C. elegans* orthologue of human Survivin, regulates transcription and development. In order to visualize the BIR-1 transcriptional function in non-dividing cells, we performed a genome wide analysis of *bir-1* loss of function and identified numerous collagen and ribosomal genes as its targets. Subsequently, we showed that genes that were repressed by *bir-1* inhibition are dramatically induced by its overexpression in transgenic animals.

I conducted or participated on most experiments described in this study with the exception of microarray experiments. The project described in this thesis was part of the greater project of the whole research group.

4 MATERIALS AND METHODS USED IN THIS STUDY

4.1 Methods used in work addressing the expression of TRs/RXRs and SKIP/Survivin in glial tumors

Biopsy samples (diffusely infiltrating astrocytomas and non-tumorous controls from patients with refractory epilepsy); Extraction of nucleic acids and preparation of protein lysates; Reverse transcription; Conventional and Real-Time PCR; Western blot analysis; Immunohistochemistry

4.2 Methods used in work addressing the expression of HDAC3 in glial tumors

The material methods (Biopsy samples; Extraction of nucleic acids and preparation of protein lysate; Reverse transcription; Amplification and characterization of HDAC3; Real-time PCR; Western blot analysis; Immunohistochemistry; Imunofluorescence, Bright field, epifluorescence and laser scanning confocal microscopy; Co-localization map construction) are described (Liby et al., 2006a).

4.3 Methods used in the study addressing the transcription role of BIR-1 in *C.elegans*

The material and methods (The *C. elegans* Strains, RNA mediated interference (RNAi), Large scale RNA interference; Extraction of nucleic acids; Reverse transcription; Microarrays experiments; Real-time PCR) are described in published studies (Liby et al., 2006a; Liby et al., 2006b).

5 RESULTS

5.1 The Expression of TRs, RXRs and Selected Putative Interacting Proteins, SKIP and Survivin, in Human Astrocytic Glial Tumors

5.1.1 The expression of TRs, RXRs, SKIP and Survivin at the mRNA level

In this study 32 biopsies of limited volume were analysed. Five samples were glial non-malignant tissues removed during therapeutic surgery, nine were low-grade astrocytomas and eighteen high-grade astrocytomas (grades III and IV). Part of the tissue was used for extraction of total RNA and reverse transcribed. The Real-time PCR method was optimalized firstly on U373 glioblastoma cell line. Secondly, the bioptical tissue had to be used for more accurate optimalization. Only the samples with melting profile characteristic for the specific amplification products were ranked. Values were normalized according to the expression of β-tubulin. Selected amplified sequences were directly sequenced and the specificity of the amplification confirmed.

Thyroid hormone receptors` cDNAs of α1, α2, β1 and β2 and cDNA`s of SKIP and Survivin were efficiently amplified from all examined cases. We observed a large diversity in the expression of selected genes in all groups of examined samples.

A trend, although not statistically significant assuming the null hypothesis, of increased TRα1 expression, especially in low grade astrocytomas, was observed. The TRα2 isoforms were found elevated in LGA. Obtained copy numbers suggests that TRα2 isoform is also the most transcribed isoform in astrocytomas. The region of TRα, common to TRα1 and TRα2, was found slightly eleveted in LGA and decreased in HGA.

Interestingly, a trend of TRβ1 expression was decreased with the severity of the disease (statistically significant, with 96% probability in student *t*-test, if controls compared against all tumors). A trend of TRβ2 expression was found, statistically not significant, elevated in astrocytomas. The region of TRβ common to TRβ1 and TRβ2 was decreased statistically significantly (95% of probability) in HGA although elevated in LGA.

RXRα, β and γ were found present in controls and tumors, with increased expression in tumors. RXRβ was the most expressed isotype. The increase of RXRγ was statistically significant ($P<0.05$) in high-grade astrocytomas.

The expression of SKIP and Survivin was found elevated in tumors. The connection of Survivin with cancer is widely documented and accepted. Also our results showed a trend of elevation in tumors (although statistically not significant in student t-test), with variable expression among studied cases.

5.1.2 The expression of TRα1, TRα2 and TRβ1 at the protein level

We prepared a collection of one non-malignant gliosis, three diffuse astrocytomas (WHO grade II), one anaplastic astrocytoma (WHO grade III) and three glioblastomas (WHO grade IV).

Different antibodies, commercial as well as antibodies obtained based on cooperation, were used. Interestingly, anti TRα1 monoclonal antibody (04/027, a kind gift from Onno Baker), detected multiple proteins with lower and higher molecular mass than predicted size of TRα1 (47,7 kD). Two low-grade samples, anaplastic astrocytoma and one glioblastoma showed a faint band between 42 to 60 kDa. These and additional bands became strongly detectable in all cases after prolongated exposure period. Multiple immunoreactive bands were detected as well in U373 glioblastoma cell line. The result was compatible with the possibility of formation of multiple protein isoforms of TRα1, but can not be taken as support of such situation as unspecific interactions could not be excluded. We started experiments for direct mass spectroscopic characterization of proteins recognized by antibody.

Immunoreactive bands of expected size of TRα2 (50,7 kDa) were detected in all samples except one diffuse astrocytoma (case 9) and control tissue (case 19).

A single band of expected size of TRβ1 (55 kD) was detected in all samples. This protein was expressed strongly in one anaplastic astrocytoma (case 11) in concordance with high TRβ1 expression at mRNA level in this case.

5.1.3 The expression of TRα1 and TRα2 at the cellular level

Eight samples (the same as in WB) were examined by histochemistry using the peroxidase method. Monoclonal anti TRα1 (04/027) and anti TRα2 (04/028) antibodies were used (the same as for WB). One control material (case 3) was evaluated as benign gliosis under the reparative process, glioma was excluded by pathologist.

Interestingly, a strong and characteristic pattern was obtained in case of TRα2. It was found predominantly in the cytoplasm, although nuclear presence was also detected. The

pattern did not differ between control tissue and tumors, but was considerably stronger in tumors.

The TRα1 immunoreactive proteins were detected abundantly in both, nuclei and cytoplasm, in all samples, but dramatically strongly in glioblastomas.

5.1.4 Functional analysis of TRs in U373 glioblastoma cell line

We searched if thyroid hormone receptors found in the U373 cell line are functional in regulation of thyroid hormone responsive genes. We trasfected U373 cells with exogenous thyroid responsive promoter, vectors without an insert or vectors coding for thryroid receptor apha 1 and RXRα and assayed the effect of T3 on expression of the reporter that codes for fire fly luciferase. For the normalization of results, a control vector coding for Renilla luciferase was used. We did two independent 48 hours experiments. Treatment with thyroid hormone increased the transcription from thyroid dependent promoter approximately by 50%. Additional cotransfection with RXRα led to further increase of the expression to approximately 80% increase. In many cell lines, such expression of transcription from exogenous throid responsive poromoters is not visible. Our results indicate that endogenous thyroid receptors are functional in U373 cells. In some studies, it was proposed that estrogen receptors may also be influenced by thyroid hormone but these would certainly not be cooperative with additional RXRs as was observed in our experiments.

5.2 Elevated and Deregulated Expression of HDAC3 in Human Astrocytic Glial Tumors

5.2.1 Characterization of HDAC3 expression in non-malignant glial tissues and glial astrocytic tumors at the mRNA level

The *HDAC3* gene (NT_029289.10), which is localized on chromosome 5, spans 16770 by and contains at least 15 exons. More than 380 partial sequences and 17 possible transcripts are deposited in public databases. There is evidence of existence of at least four types of HDAC3 transcripts that contain open reading frames. The HDAC3 isoforms deposited in databases can be represented by four sequences: the basic isoform, known as HDAC3 (Yang et al., 1997), which is represented in this work by AF039703.1 yielding a 1920 by long open reading frame coding for a 428 amino acid protein with a calculated molecular weight of 48.8 kDa; HDAC3 isoform A (U75696.1) that has a 1941 by long CDS, coding for a protein with 429 amino acids and a

calculated molecular weight of 49.1 kDa; HDAC3 isoform C (AF005482.1) with 1981 by long CDS, coding for a protein with 371 amino acids and a predicted molecular weight 42.3 kDa; and a short isoform lacking the N-terminal half of the CDS, represented by AF 130111.1. For the purpose of this study, we named the shortest isoform HDAC3 isoform D.
The coding region of isoform D starts in the 7th exon and uses the methionine that is present in all isoforms. The protein sequence derived from the open reading frame of isoform D is composed of 223 amino acids (calculated molecular weight 25.6 kDa) that form the C-terminal part of all remaining isoforms. Isoform D lacks the N-terminal sequence that was shown to be indispensable for histone-deacetylating function of HDACs (Zhang et al. 2005).

We prepared a collection of four glial non-malignant tissues removed during therapeutic surgery, six low-grade gliomas and eleven high-grade gliomas (grades III and IV). Frozen sections were cut using a Leica Cryocut II apparatus and stored in eppendorf tubes. Total RNA was prepared and cDNA was made using Superscript II reverse transcriptase and random hexamers as primers.

Primers were designed to amplify HDAC3 and HDAC3A (from the start of a shared region to the stop codon), HDAC3C and HDAC3D. Similarly, primers for the amplification of the region common to all four isoforms were designed . Selected amplified sequences were characterized by direct sequencing and specificity of the amplification confirmed for all studied isoforms. Since the two longest isoforms are likely to be functionally similar if not identical, we searched for the expression of both HDAC3 and HDAC3A in all examined samples. PCR targeted to region B amplified the expected fragments from all examined samples and the specificity was confirmed by sequencing .

Quantitative PCR directed at the region common to all four isoforms also amplified the expected fragments from all samples and the specificity of the amplification was confirmed by direct sequencing. For quantitative PCR, the standard curve for increasing number of copies per reaction was determined together with the assayed samples and the threshold set at the start of the efficient amplification. With the exception of one case that was histologically classified as grade II glioma, but was recurrent, all non-malignant glial tissues and grade II gliomas showed less than 10,000 copies per 150 ng of total RNA. Contrary to that, seven out of 11 gliomas of grade Ill and grade IV yielded more than 24,000 copies per 150 ng of total RNA. Interestingly, four of six examined gliomas of grade II had levels lower than those found in non-malignant tissue in 150 ng of total RNA.

Analysis of HDAC3 expression compared to the histological type of the examined tissues supported the elevated HDAC3 expression in high-grade gliomas. Expression of HDAC3

followed the expression of two housekeeping genes, glycerol 3-phosphate dehydrogenase, and ß-actin, in keeping with the possibility that HDAC3 elevated expression is part of an overall disturbed expression profile in glial tumors. Normalization against ß-tubulin alone followed the pattern of elevated HDAC3 expression in total RNA. The relative level of HDAC3 expression compared to ß-tubulin also indicated elevated levels of HDAC3 in high-grade gliomas. A similar trend was observed for quantitative analysis of the expression of all three long isoforms or two longest isoforms (HDAC3 and HDAC3A), indicating expression of multiple isoforms of HDAC3 in high-grade gliomas.

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5.2.2 Detection of HDAC3 expression at the protein level by Western blot analysis

Western blot analysis with a rabbit polyclonal antibody revealed a faint band corresponding to a protein with an approximate size of 50 kDa in non-malignant glioses but prominent expression in tumour samples. Two non-malignant samples had a prominent band at 25 kDa (NCH19, 20) that presumably corresponds to a protein of N-terminally abrogated isoform D. On longer exposure films, a band with a size of approximately 58 kDa was also observed. Contrary to that, three out of five gliomas of grade II displayed 2-fold increased expression of the 50 kDa protein compared to the mean values seen in non-malignant samples and two expressed a 25 kDa HDAC3 immunoreactive protein. All nine examined gliomas of grade III and IV expressed the 50 kDa HDAC3 and five of the nine gliomas had 2-fold the levels found in non-malignant tissue. Two samples contained a strongly expressed 48 kDa protein and a 42 kDa protein. Four out of nine cases had elevated levels of a 25 kDa protein. Densitometric analysis from two analyses prepared from the same material for each case revealed elevated expression of 38-50

kDa proteins in high-grade tumours. Analysis of HDAC3/HDAC3A and HDAC3C expression related to the histological type of assayed tissue showed an increasing trend of HDAC3 expression in high-grade gliomas, indicating significantly elevated expression of the three longest isoforms in tumours and a trend for higher expression of HDAC3 in high-grade tumours. The result was significant in the Student's t-test with 95 % probability.

In order to know whether HDAC3 is inherent to glial cells and not to other cell types that may contaminate the tumour samples, we studied HDAC3 expression in the cultured U373 human glioblastoma cell line. Quantitative PCR yielded values that corresponded to the highest values found in tumours. Similarly, HDAC3 at the protein level was found to be expressed in U373 cells as a prominent 50 kDa protein. Contrary to that, the 25 kDa protein was not observed in U373 cells despite the fact that the short isofotm HDAC3D was amplified from U373 cells and confirmed by sequencing.

5.2.3 Analysis of HDAC3 expression at the cellular level

Thirty-five tumours were examined by histochemistry using the peroxidase method and by immunofluorescence. Immunohistochemistry detected HDAC3 weakly in the cytoplasm and nuclei in normal brain glial cells as well as in gliosis and low- and high-grade gliomas. For an orientation analysis, all samples were evaluated as described in Methods. Cases where the observer classified 50 % of cells as cells containing cytoplasmic or nuclear staining that differed from control sections were classified as potentially HDAC3-positive. All evaluated tumours fulfilled this criterion.

For immunofluorescence, the samples were processed and evaluated as described in Methods. Nonmalignant glial cells were weakly stained predominantly in the nuclei. Non-malignant gliosis showed stronger labelling for HDAC3 both in the nuclei and the cyto-plasm. High-grade gliomas were strongly stained in the cytoplasm and had a pronounced focal character, containing areas with prominent cytoplasmic staining and areas with stronger nuclear HDAC3 localization. Although many fields examined were clearly characterized by observers as nuclear HDAC3 fluorescence,
a partial cross-talk from DAPI channel could not be excluded using the epifluorescence band pass filter sets. Analysis using the criterion of 50 % subjectively positive cells indicated that all cases of glial tumours were positive for HDAC3 staining.

Next, we analysed whether U373 cells showed staining for HDAC3 similar to the cells in the examined tumours. Epifluorescence detected HDAC3 in the cytoplasm of all

cells. Nuclei were also stained but in some nuclear staining seemed to be compared to the cytoplasm.

Confocal microscopy

We employed confocal microscopy in order to evaluate the co-localization of HDAC3 staining with the nuclear compartment strongly labelled by DAPI. Analysis of U373 cells showed that HDAC3 staining was clearly detectable throughout the cytoplasm and was accumulated around the nucleus in many cells. Nuclear staining was also clearly detectable. Co-localization maps using single pixel overlap coefficient values were calculated as described in Material and Methods.

Co-localization maps revealed nuclear HDAC3 staining in all examined nuclei.

Detection of HDAC3 expression in human glial tumors by confocal microscopy

A collection of samples that included all cases studied biochemically (except for three non-tumorous glioses that were not available as paraffin-embedded material) was analysed as described in Methods. Analysis confirmed the HDAC3 expression pattern detected by immunohistochemistry and epifluorescence in the cytoplasm.
A low and relatively uniform pattern was observed in normal glial tissue and in non-malignant gliosis, more intensive labelling in low-grade gliomas and strong focally accented labelling in high-grade gliomas. The nuclear labelling was also confirmed in all areas studied. Co-localization maps clearly showed nuclear localization of HDAC3 in all examined tumours.

5.3 Analysis of The Regulatory Potential of Bir-1 (The Orthologue of Human Survivin) in Regulation of Transcription in *C.elegans*

5.3.1 Inhibition of the bir-1 causes decrease in expression of several proliferative genes in L1 larvae

The effect of *bir-1* inhibition on L1 larvae

Embryos of wild-type hermaphrodites were prepared using a standard protocol and synchronized L1 larvae were prepared. Synchronized cultures were incubated on a bacterial lawn of control E. *coli* transformed with vector L4440 carrying the cloning site and a non-specific sequence flanked by bacterial promoters on both sides. For *bir-1* inhibition, a 350 by sequence with the majority of *bir-1* cDNA was used. The production of dsRNA was induced by IPTG in both control and *bir-1*-inhibited cultures. Worms treated with *bir-1* or control RNAi did not differ visibly in the time of L1/L2 molt. Only a slight dpy phenotype was observed in a proportion of bir-l-inhibited worms. The development of seam cells that divide at the end of L1 stage was monitored in JR667 and SU93 transgenic larvae L1 prepared similarly as wild-type larvae. These experiments showed that inhibition of *bir-I* by the feeding method does not affect cell divisions of seam cells.

bir-1 inhibition by RNA interference induces whole-genome transcription changes

To determine the transcription profile by microarrays, three independent experiments were done and evaluated as three control versus *bir-1* RNAi pairs. The statistical analysis labelled the results as Present/Absent (P, A), Increased/Decreased (I, D), No Change (NC), Moderate Increase (MI) and Moderate Decrease (MD). The detection and change P values of the absolute majority of data indicated marginal differences in the analysis of the data. One set of experimental data showed a major problem in evaluation or preparation of the experiment and was eliminated from the analysis. The computer program recognized 67 annotated spots as decreased in both evaluated *bir-1* RNAi experiments and 226 annotated probes as increased in *bir-1* RNAi experiments.

Interestingly, genes recognized as decreased were mostly found in groups of genes that showed highest readings on Affymetrix microarrays. The analysis of values obtained by Affymetrix microarrays showed that the group of 30 gene probes with highest values

showed decreased readings in *bir-1* RNAi while the genes with average readings showed both increased and decreased or unchanged values in *bir-1*RNAi-treated cultures.
The tendency of decreased readings in RNAi-treated cultures for genes with highest values on Affymetrix microarrays were not observed in our unrelated microarray experiments.

Genes that were identified as genes inhibited in *bir-1* RNAi included several collagen genes and genes with a known dpy phenotype: *dpy-2, dpy-3, dpy-4, dpy-7, dpy-8, dpy-9, dpy-10, dpy-13, dpy-15, dpy-17, col-1, col-3, col-10, cot-117, col-92, col-93, col-94, cot-125, col-144, co1-154, col-160, col-166, col-167, col-169,* genes for several ribosomal proteins: *rps-1, rps-4, rpl-2, rpl-21* and enzymes (e.g. phosphoheptose isomerase).

Next, we amplified coding regions of selected collagen genes that were identified by microarray analyses and selected genes that were suitable for quantitative PCR according to the amplified fragment (that was confirmed by direct sequencing) and the character of the melting curves. Ten collagen genes fulfilled these criteria and were used for further analysis: *dpy-2, dpy-4, dpy-7, dpy-8, dpy-13, col-94, col-125, col-144, col-166,* and *col-167.* Manual evaluation of raw data obtained using Affymetrix microarrays indicated a 20 to 40 % decrease in the expression in *bir-l*-inhibited worms.

Quantitative RT-PCR confirmed the decreased expression of all evaluated collagen genes. However, the expression of several collagen genes that were identified by microarrays as genes inhibited in *bir-1* RNAi-treated larvae could not be quantified because unspecific sequences were amplified as indicated by multiple bands detected in the final PCR products and by the character of the melting curves.

5.3.2 Overexpression of BIR-1 has the potential to dramatically upregulate developmentally active collagen genes

bir-1 overexpression induces a dramatic increase in the expression of collagen genes whose expression is sensitive to *bir-1* RNAi during normal development

In order to see whether genes that showed decreased expression in *bir-l*-inhibited larvae are also sensitive to elevated levels of BIR-1, we generated transgenic lines expressing *bir-1* mRNA from a transgene regulated by a heat-shock inducible promoter and confirmed the presence of the transgene by direct sequencing. A short heat-shock induction (30 min at 34°C) strongly induced the expression of all ten examined collagen genes normalized to the

expression of actin *(act-1)*. This induction and an almost identical expression pattern were observed in experiments normalized according to the expression of a large subunit of Pol *II, ama-1*. Interestingly, the expression of transcripts used for the normalization of results *(act-1* and *ama-1)* was elevated in experiments arranged to determine the number of amplified copies in equal amounts of starting material (based on total RNA), indicating that the observed difference in the expression of collagen genes may actually be larger.

6 DISCUSSION

In this study we characterized the expression pattern of the TRs/RXRs genes and selected putative interacting proteins in astrocytic brain tumors.

We found that four major TR isoforms are expressed in diffusely infiltrating astrocytomas and benign gliosis. Three RXRs genes are expressed in benign gliosis as well as in tumors.

Our data show that the expression pattern of these NRs is dramatically deregulated, with variable levels of expression in tumors of the same grade. Clearly, in some tumors TRs and RXRs are strongly elevated.

Thyroid receptors are suspected to be important factors in development of many malignancies since their discovery which showed that thyroid receptors are cellular holmologues of v-erb A (Sap et al., 1986; Weinberger, 1986). Numerous studies attempted to find mutated forms of TRs in cancers. It is concluded that mutations and deletions are not a frequent event in cancers (Gonzales-Sancho, 2003). Some studies indicated, however, that decreased expression of throid receptors, namely TRβ1 may be caused by methylation of the TRβ1 gene in breast cancers (Li et al., 2002).

Furthermore, the suspicion that TRs could be implicated in carcinogenesisis, may be reflected by observations made on transgenic animals defective for specific isoforms of thyroid receptors. While mice expressing the v-erb A develop hepatocellular tumors (Barlow et al., 1994), no higher incidence of malignancies is reported for TR Knock-Out animals (Forrest and Vennstrom, 2000).

Initial analysis of expression of steroid and thyroid receptors in several brain tumors and glial cell lines indicated that these genes are commonly coexpressed in brain tumors (Magrassi et al., 1993), but the expression of thyroid and retinoid receptors in glial tumors is not available. The findings presented in this study suggest, that thyroid receptors are likely to be part of the oncogenesis of glial tumors.

Our results show that HDAC3 is expressed at high levels in malignant human astrocytic glial tumors. HDAC3 was found to be expressed in multiple isoforms on both mRNA and protein levels. Four different transcripts are listed in the expert-curated NCBI database and more than 17 transcripts were found in more than 130 types of HDAC3 ESTs.
The originally reported HDAC3, HDAC3A and HDAC3C were amplified using specific primers and reverse transcription-PCR and their sequences were confirmed by direct sequencing. Primers designed to amplify the N-terminally deleted HDAC3D mRNA led to the amplification of the expected fragment in one case but not in the remaining cases. HDAC3D was also found to be expressed in the U373 glioblastoma cell line on the mRNA level but surprisingly not on the protein level.

In keeping with the amplified cDNAs for the three long isoforms of HDAC3, Western blots detected proteins with sizes corresponding to the expected proteins derived from HDAC3, HDAC3A and HDAC3C. In some cases, a protein of approximately 25 kD was detected by HDAC3 C-terminus specific antibody and can be expected to correspond to HDAC3D. In most cases that expressed the 25 kD protein, primers designed to amplify a 183 bp long fragment amplified longer fragments, ranging from 400 to 600 bp. Sequencing showed that at least in some cases, these fragments contained the coding sequence of HDAC3D.
Since HDAC3 and HDAC3A differ only in 15 or 16 amino acids, respectively, it is likely that their functions and cellular regulations are very similar if not identical. HDAC3C differs from the two longer isoforms by a deletion of 58 (57 compared to HDAC3A) N-terminal amino acids, but can be expected to be a functional HDAC based on a comparison to domains shown to be irreplaceable for the histone deacetylase function (Zhang et al., 2005; Zou et al., 2006). The shortest isoform, denominated HDAC3D, may correspond to the 25 kDa protein that is clearly recognized by Western blot in approximately one half of the cases in this study. It is unlikely, that HDAC3D is a functional histone deacetylase, since it is missing domains necessary for histone deacetylase activity and can be expected to function as a negative regulator of some HDAC3 isoforms or even multiple histone deacetylases. Since our analyses failed to amplify the predicted cDNA of HDAC3D in several cases that expressed the 25 kDa protein recognized by HDAC3 antibody, its existence needs to be confirmed by direct proteomic methods.

The findings of this study indicate that the mRNA of HDAC3 is elevated in tissues of high grade gliomas compared to non-malignant gliosis and low grade gliomas. This was observed using quantitative PCR directed to amplify the region that is common to all four

isoforms, three long isoforms and two longest isoforms from equal amount of total RNA extracted from tissues. We interpret these findings as increased presence of HDAC3 in high grade glioma tissues but not necessarily per cell or nucleus. The high expression of HDAC3 in malignant tumors was supported by the expression of HDAC3 on protein level detected by Western blots.

Detection of HDAC3 by commercial antibodies and fluorescent microscopy (epifluorescence as well as confocal microscopy and colocalization analyses) show rather low nuclear expression of HDAC3 in histologically normal glial tissue. Contrary to that, gliomas, especially high grade gliomas showed strong cytoplasmic HDAC3 expression. Confocal microscopy and colocalization maps detected HDAC3 in nuclei of an absolute majority of cells of glial tumors examined. Our data are in keeping with the possibility that HDAC3 may be indispensable for proliferation of glial cells and may play important roles in the malignant transformation of glial cells and growth of astrocytic tumors. This is supported by findings that inhibition of histone deacetylation by histone deacetylase inhibitor 4-phenylbutyrate, exerts anti-proliferative and differentiation inducing effects on a glioblastoma cell line that is connected with the induction of connexin 43 expression and inter-cellular communication via gap-junctions. Also, the non-phosporylated forms of connexin 43 and glial acidic fibrillary protein were induced by 4-phenylbutyrate (Asklund et al., 2004). HDAC3 is an effector in the transcription repression by thyroid receptors not occupied by ligand (Ishizuka and Lazar, 2003). Cheng and coworkers have shown that cyclin D1 can bring HDAC3 into the complex of thyroid receptors in a ligand independent manner thus overruling the transcription activation function of liganded thyroid receptors. Thus, the nuclear HDAC3 localization may be expected to have a potency to specifically inhibit a number of differentiation pathways.

An interesting feature observed in our experiments was the focal character of elevated cytoplasmic HDAC3 expression.

Histone deacethylases were shown to function not only in the nucleus, but also in the cytoplasm. HDAC inhibitors disrupt the complex between HDAC/PP1 and in consequence dephosphorylation of Akt I U87MG gliolbastoma and PC-3 prostate cancer cells. The HDAC inhibitors that were assayed differed in potency to activate the Akt dephosphorylation. TSA showed the highest effect compared to HDAC42 and SAHA (Chen et al., 2005). The proposed mechanism includes interaction of HDAC with protein phosphatase 1 (PP1) in an active, phosphorylated form and excludes HDAC from interaction with other proteins, including Akt. Akt was shown to interact with HDAC1 and HDAC6 (Chen et al., 2005). HDAC3 activity, phosphorylation status and cellular localization were shown to depend on

the interaction with PP4 (Gao et al., 2005). The activity is dependent not only on the phosporylation status, which may regulate the interaction of class II HDACs with 14-3-3 proteins (Bertos et al., 2001; Gagnon et al., 2003; Grozinger and Schreiber, 2000), but also on the activity of corepressors of transcription factors (Guenther et al., 2001; Ishizuka and Lazar, 2003). It was recently shown that HDAC3 is also localized at the plasma membrane and may be phosphorylated by Src (Longworth and Laimins, 2006). HDAC3 expression, activity and cytoplasmic localization is connected with TNF signaling. HDAC3 inhibits the activation of MAPK11-mediated activating transcription factor-2 and expression of TNF (Mahlknecht et al., 2004). In the opposite way, regulation of nuclear translocation of HDAC3 by IκBα is required for the inhibition of PPARγ function by TNF (Gao et al., 2005).

In this study, we found HDAC3 in both nuclear and cytoplasmic localizations in all examined cases of high grade gliomas. It can be speculated that the dual deregulated expression of HDAC3 may be important for biological properties of malignant gliomas.

Experiments included in this thesis show that BIR-1, a homologue of the human cancer related protein Survivin, regulates expression of several developmentally active collagen genes during the normal development of C. elegans and has a strong potential to affect gene expression when expressed at non-physiological levels in transgenic animals.

C. elegans offers a versatile model for studies of transcription regulation in both dividing and non-dividing cells. The development of C. elegans proceeds through an embryonic stage and four larval stages. Adult C. elegans worms have a constant number of somatic cells, 956 in hemaphrodites and 1031 in males. The cell lineages follow a developmental scheme that result in the formation of specialized tissues and cells in a tightly regulated pattern. At the end of the embryonic stage, the newly hatched L1 larva has approximately 550 cells, and the subsequent cell divisions in the L1 stage form an animal that has most cells developed. Only a limited number of cells continue to divide during a narrowly framed time window. Seam cells, specialized hypodermal cells that form two lines on the side of the larvae asymmetrically divide once in each larval stage and form a new seem cell that keeps the blast cell character and a hypodermal cell that fuses with the lateral hypodermal syncytium. Proper development of seam cells is critical for normal body shape and motility.

Most cells can be regarded as postmitotic in the larval stages of C. elegans and the separation of dividing and non-dividing cells offers a system for studies of protein function related to cell division and postmitotic functions.

BIR-1 and Survivin execute critical functions during cell division: the regulation of the spindle formation and the proper separation of chromosomes. These functions are dramatic

and since the cell cycle projects to complex cellular events including transcription regulation may obscure non-mitotic events. Inhibition of BIR-1 and Survivn function leads to mitotic arrests, defects in karyokinesis and cytokinesis including the formation of aneuploid cells and nuclei-free cell bodies. The other functions of Survivin include potent inhibition of apoptosis and a newly discovered role in the regulation of stress response.

Our previous work identified a new function of BIR-1, that is not related to mitotic functions: the regulation of transcription and development in non-dividing cells (Kostrouchova et al., 2003a). Structural data that are available for Survivin indicate its wide ability to interact with other proteins. Survivin is small 123 aa protein that forms a zinc finger BIR domain and a laterally postioned helix. Both domains are likely to effectively bind other proteins including Bir1 itself that was in crystalographic studies found unexpectadley dimerizing through the region that forms a tip of triangularly shapd molecule. Survivin and BIR-1 were shown to form complexes with more than one protein. BIR-1 interacts with a small protein CSC-1 (Romano et al., 2003) and INCENP (Romano et al., 2003) and together with both proteins is involved in docking the Aurora B kinase Air-2 to chromosomes (Speliotes et al., 2000).

The binding function of Survivin includes binding of caspases and interaction with mitochondrial proteins. It seems likely that the interaction has inactivating consequences. The antiapoptotic functions seem to have a wide spread role in most human cancers. The high expression of Survivin is generally linked to a higher malignancy and progression in most cancer types. Both Survivin and BIR-1 were shown to reside both in the cytoplasm and in the nucleus. While the cytoplasmic expression of Survivin is reported as an unfavorable criterion in most studied cases, the nuclear expression was found to be related to a better prognosis.

Despite that Survivin is generally found to be overexpresssed in most cancers, its elevated expression in transgenic mice does not induce cancer formation itself but elevates the sensitivity to cancer promoting events and is connected with changes of gene expression in microarrays.

The structure and potentially multiple protein interaction potential of BIR-1/Survivin suggests that its elevated expression and spacially restricted presence may induce fundamental changes in the availability of regulatory proteins and may change the capacity of interacting proteins to participate in protein complexes. Since BIR-1 is able to bind Air-2 and phosporylate histones H3 on P10, we hypothesized that kinase docking may be a part of BIR-1's transcriptional role in non-dividing cells. Histone H3 phosphorylation is the critical event for chromatin compaction during mitosis which is generally connected to the silencing of

transcription but may also be linked to transcription activation. Phospohoacetylated histones H3 in the form of K9-Ac S10-P and S10-P K14-Ac were found to be part of transcription activation and it was suggested that phosphorylation of histone H3 may even precede the acetylation and may actually be the starting event in transcription activation (Hauser et al., 2002). Alternativelly, both phosphorylation and acetylation events may be functionally separated and project synergistically but independently to the promoters of genes (Thomson et al., 2001). The phosphorylation of histone H3 may be connected to regulatory events that exceed modification of chromatin events similarly as acetylation of histones that represent a modification generally related to transcription activation, is part of posttranslational modifications of both chromatin proteins and transcription factors as shown for nuclear hormone receptors (Lefebvre et al., 2002; Lo et al., 2000; Nowak and Corces, 2000). Phosphoacetylation of histones in nucleosomes regulated by c-fos and c-jun proteins was shown to be part of transcription activation (Clayton et al., 2000).

The role of Survivin in the regulation of gene expression was also observed in mammalian systems. Survivin enhances the level of p21ras mRNA as well as its activated form on the protein level (Temme et al., 2005). Survivin regulates the expression of p53 and its protein family members both on mRNA and protein levels (Wang et al., 2004). The involvement of Survivin in the regulation of gene expression via the up-regulation of SP-1 mediated gene transcription was shown on colon cancer cells (Asanuma et al., 2004).

The results reported here suggest that BIR-1 may be involved in contacts with multiple transcriptionally active proteins. As reported here, *bir-1* inhibition repressed transcription of most but not all genes that showed the highest transcriptional activity in microarrays and decreased the values recorded for 67 genes. A larger group of genes comprising 227 members was identified as genes potentially transcriptionally inhibited by BIR-1. Together with the findings that most transgenes and transfected reporters are more transcriptionally active in presence of BIR-1, this suggests that BIR-1 is likely to activate the ongoing active transcription. Multiple contacts of BIR-1 with transcriptionally active proteins may project to elevated expression of specific genes in *bir-1* RNAi.

In conclusion, the data reported here support the active role of BIR-1 in transcription regulation during *C. elegans* development and indicate that BIR-1 interferes with transcription regulation when expressed at high levels. BIR-1 and Survivin can substitute for each other in several systems (Speliotes et al., 2000). It is therefore likely that Survivin, strongly overexpressed in tumors, has profound effect of cancer specific transcriptome.

7 CONCLUSIONS

Our results document that TRs/RXRs are expressed and deregulated in human astrocytic tumors.

Cofactors that have potential to modulate the regulatory functions of thyroid receptors, namely SKIP, Survivin and HDAC3 are elevated and deregulated in human astrocytic tumors.

The involvement of BIR-1 in regulation of transcription of developmentally active genes confirmed.

8 LIST OF AUTHOR`S PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Libý P., Kostrouchová M., Pohludka M., Yilma P., Hrabal P., Sikora J., Brožová E., Kostrouchová M, Rall JE, Kostrouch Z (2006) Elevated and Deregualted Expression of HDAC3 in Human Astrocytic Glial Tumours . Folia Biol 52, 21-33

Libý P., Pohludka M., Vohanka J., Kostrouchová M., Kostrouch D., Kostrouchová M., Rall J.E., Kostrouch Z. (2006) BIR-1, the homologue of human Survivin, regulates expression of developmentally active collagen genes in C.elegans. Folia Biol 52, 101-8

PRESENTATIONS

Libý P., Pohludka M., Yilma P., Hrabal P., Kostrouchova M., Kostrouch Z. (2005) Regulation of gene expression by thyroid receptors in human glial tumors. Oral presentation. *Kunců v memoriál, Neurochirurgická klinika 1.LFUK a UVN,Praha,24.-25.03.2005*

Libý P., Pohludka M., Yilma P., Hrabal P., Kostrouchova M., Kostrouch Z. (2005) Regulation of gene expression by thyroid nuclear hormone receptors in human glial tumors. Oral presentation. *6th Student Scientific Conference. 1st Faculty of Medicine , Charles University in Prague, 23.05.2005*

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