Survivin products in pituitary tumors

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Abstract

OBJECTIVES: Survivin is a member of the inhibitor of apoptosis protein family, which was recently showed to be expressed by different benign and malignant human tumors. Still very little is known about survivin expression in pituitary tumors. In spite of the fact that pituitary tumors in histological examination are usually benign, in the clinical process a certain number of pituitary adenomas is capable of aggressive growth, recurrence and invasion of the surrounding structures. The aim of the present study was to assess the presence of survivin transcripts and protein in different types of pituitary tumors and to evaluate survivin expression levels in invasive and non-invasive pituitary tumors.

DESIGN AND METHODS: The analyzed material consisted of tumor tissue samples obtained during standard neurosurgical removal of the tumor from 23 patients in whom acromegaly (n=14), non-functioning pituitary tumor (n=6), prolactinoma (n=2) and corticotropinoma (n=1) were diagnosed. As a control of the study normal pituitary tissue obtained at autopsy was used. Amplification of survivin gene using sequence specific primers and qRT-PCR method and immunohistochemical staining with primary polyclonal antibodies against human survivin were performed.

RESULTS: Our study demonstrated the presence of survivin mRNA in all 23 analyzed pituitary tumors. Survivin expression was also observed in normal pituitary, but the level of its expression was 6-fold lower than in tumors tissue when studied by real time RT-PCR. The difference between the levels of survivin expression in invasive and non-invasive tumors was not statistically significant. Immunohistochemical analyses revealed the presence of the protein in both normal and tumor tissue of pituitary. Immunostaining of tumor tissue was not uniform. Survivin was observed mainly in the nuclei of cells collected in clusters. The presence of the protein in normal pituitary was restricted to small population of cells.

CONCLUSIONS: The present study showed that overexpression of survivin is characteristic for pituitary tumors. Further analysis of this protein expression profile should demonstrate whether survivin might be use as a prognostic marker in diagnosis and therapy of pituitary adenomas.

ORIGINAL ARTICLE

Abbrevations & Units:
IAP – inhibitor of apoptosis protein
BIR – Baculovarius IAP repeat
mRNA - messenger ribonucleic acid
cDNA – complementary deoxyribonucleic acid
PCR – polymerase chain reaction
qRT-PCR – quantitative polymerase chain reaction
HPRT - hypoxanthine phosphoribosyl transferase
PTTG - pituitary tumor transforming gene

INTRODUCTION

Survivin belongs to the IAP family, which consists of anti-apoptotic proteins. Survivin suppresses programmed cell death and stimulates the cell division since its involved in the cell cycle progression and microtubule stability [1,2,3,4]. This protein is present in fetal and neoplastic tissues, but is not detected in most adult differentiated tissues [5]. Survivin gene spans 14,7 kbp on the chromosome 17 and is localized in band q25 [6]. Structurally, it contains a single Baculovarius IAP Repeat (BIR) domain and an extended α-helical coiled-coil C-terminus but does not contain a RING-finger domain, found in other IAP's members [2]. It is suggested that survivin inhibits apoptosis of tumor cells, and accelerates their mitotic activity [7,8]. Survivin expression was detected in G2/M phase of the cell cycle. The protein associates with microtubules of the mitotic spindle and begin the mitosis. Disruption of this interaction results in the loss of its anti-apoptotic function [7]. Recent study showed that various human benign neoplasms such as breast adenomas, Bowen's disease, colon polyps and benign tumors derived from nervous system expressed survivin [9,10]. In malignant tumors survivin expression correlated with poor prognostic parameters, local recurrence and shorter disease-free survival [11,12,13,14,15,16]. Therefore it was suggested that survivin is a negative prognostic marker of majority of human cancers. Expression of survivin in brain tumors has been reported previously, however its presence in pituitary tumors is not well recognized. Pituitary tumors in majority are benign monoclonal adenomas derived from a single mutant cell and represent about 10% of intracranial neoplasm [17]. Still a certain number of pituitary adenomas is capable of aggressive growth, recurrence and invasion of the surrounding structures.

The result of present study expands our previous research showing survivin expression in pituitary adenomas [18]. This study documents the presence of survivin mRNA and protein localization in different types of pituitary tumors and in normal pituitary tissue and reveals that relative levels of survivn expression vary between normal and tumor tissues.

MATERIALS AND METHODS

Pituitary tumor tissues were obtained from 23 patients during standard neurosurgical removal of the tumor. Patients were treated with the surgery at the Department of Neurosurgery in Poznan University of Medical Science in 2007. The studied group consisted of 11 females and 12 males (mean age 45 years, range 30-61). 14 out of 23 patients have been diagnosed with GH-producing pituitary adenomas. 6 out of 23 patients have been diagnosed with nonfunctioning pituitary tumors, 2 out of 23 patients with prolactin-secreting pituitary tumors and in 1 case adrenocorticotropin–producing pituitary tumor was identified. According to the histolopathological examination the studied group was divided into two subgroups: invasive pituitary tumors (15 cases) and non invasive pituitary tumors (n=7). As a control for the research normal pituitary tissue (n=2) obtained at autopsy was used. Directly after surgical removal tumor tissues were placed in RNA*Later* (Sigma Aldrich, St. Louis, MI, USA) or fixed in 4% paraformaldehyde.

The study was approved by the ethics reviewed board of Poznań University of Medical Sciences and all patients participated after written consent.

RNA extraction

Total RNA was isolated from the tissue with TRIPure Reagent (Roche Diagnostics, Mannheim, Germany), according to manufacture protocol. 2 μg of RNA (DNase treated) was employed individually for one reverse transcription reaction in a mixture containing: 50 pmoles of survivin sequence specific primer: 5'- GGC CTC AAT CCA TGG CAG C -3' (nucleotides 438-457, GenBank AC# AF077350) or 100 pmoles oligo$(dT)_{10}$ primer, 5U/μl Expand Reverse Transcriptase, 1x Expand Reverse Transcriptase buffer, 2 mM of dNTPs, 20U RNase Inhibitor. The reaction mixture was incubated at 55^0C for 30 min, and the reaction was stopped heating probe in 85^oC and by putting on ice. All compounds used for cDNA synthesis were obtained from Roche Diagnostics, Mannheim, Germany.

PCR amplification

A 424 bp fragment of survivin was amplified from cDNA using following sequence specific primers: sense 5'-CAT GGG TGC CCC GAC GTT-3' (nucleotides 24-41, GenBank AC# AF077350) and antisense 5'- GGC CTC AAT CCA TGG CAG C -3' (nucleotides 438-457, GenBank AC# AF077350). The primers were designed to be complementary to the splice junction.

The amplification was performed in a reaction mixture containing: 1x Taq DNA polymerase buffer, 2.5 mM $MgCl_2$, 200 nM dNTPs, 250 nM of each primer and 1 unit of Taq DNA polymerase, with thermal profile as followed: 5 min at 95°C, 1 min at 95°C, 45 sec at temperature specific for the primer's set, 1 min at 72°C for 30 cycles. All compounds used for DNA synthesis were obtained from Bioline, London, UK. The ampli-

fied products (half of the reaction mixture) were electrophoresed on 1% agarose gel (FMC BioProducts, Rockland, Maine, USA).

Quantitative RT-PCR

To quantify the amount of survivin and *HPRT* (internal control) in studied tissues after RNA isolation, reverse transcription real-time PCR with LightCycler 2.0 (Roche Diagnostics) and LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) was performed. 20µl of PCR reaction mixture containing: 2.5mM of $MgCl_2$, 2 µl of SYBR Green 1 mix, 1.5 µl of cDNA and a 0.5µM of each primer. Survivin primers sequence was as described above and *HPRT* primers were designed as follows: sense – 5'-TGA AGA GCT ATT GTA ATG ACC AGT C-3' and antisense – 5'-CAA ATC CAA CAA AGT CTG GC-3' [GenBank AC# NM_000194]. The HPRT specific primers were complementary to the splice junction. The amplification program consisted of 1 cycle of 95°C with a 10 min hold, followed by 45 cycles of 95°C with a 10 s hold, annealing temperature at 68°C (survivin) or 54°C (*HPRT*) with a 5 s hold, and 72°C with a 9s for HPRT and 18s for survivin hold. This was followed by melting curve analysis, which ran for 1 cycle at 95°C with a 0 s hold, 65°C with a 15 s hold, and 95°C with a 0 s hold at the step acquisition mode.

All experiments were performed in triplicates. PCR efficiencies were calculated from the standard curves (generated using serial dilutions of *in vitro* generated transcripts) for survivin and *HPRT*. Melting curve analysis (LighCycler software package) was applied to ensure the specificity of the PCR reaction. A relative expression level of survivin gene was normalized with *HPRT*.

Immunohistochemistry

Paraffin sections of the analysed tissue were fixed in 4% paraformaldehyde and used for immunohistochemical detection of survivin. Antigens were retrieved by microwave activation in citrate buffer (10 mM, pH 6.0). After being blocked in a TBS blocking buffer, pH 7.5 (containing 100 mM TRIS-HCl, 0.9% NaCl, 0.05% Tween-20 (TBS-T) and 3% BSA) the sections were incubated overnight at 4°C in a humid chamber with primary polyclonal antibodies against survivin (BIOTREND Chemikalien GmbH, Cologne, Germany) diluted 1:200. After washing for detection of antigen-primary antibody complex EnVision+System-HRP (DAKO Cytomation) was applied. Control experiments included reactions carried out under identical conditions except for the primary antibodies that were replaced by a non-immune serum. Detection of antigen-antibodies complex was visualized employing light microscope (Zeiss, Axioskop 2).

RESULTS

Pituitary tumors express survivin mRNA

To verify at the molecular level the presence of survivin in pituitary tumors, total RNA was isolated from tumorous tissue of patients, reverse transcribed, and a 424 bp fragment corresponding to survivin nucleotides 24-457 [GenBank: AC# AF077350] was amplified. In all 23 samples of pituitary tumors as well as in 2 samples of normal pituitary survivin transcripts were detected. A representative example of the RT-PCR data is shown in Figure 1 where it is evident that we were able to amplify a specific fragment of survivin.

Relative expression of survivin in pituitary and pituitary tumors

Further quantitative analysis showed that expression level of survivin differs in normal pituitary and pituitary tumors. The relative expression of survivin in pituitary tumors was about 6-fold higher in comparison with normal pituitary. The difference between the level of survivin expression in invasive and not invasive tumors was not statistically significant and can not be correlated with histological examination of pituitary tumors (Figure 2).

Survivin protein is synthesised by pituitary and pituitary tumors

To verify the presence of survivin at the protein level and to address localisation of the protein in neoplastic tissue, immunohistochemical analyses were carried out with primary antibodies against survivin.

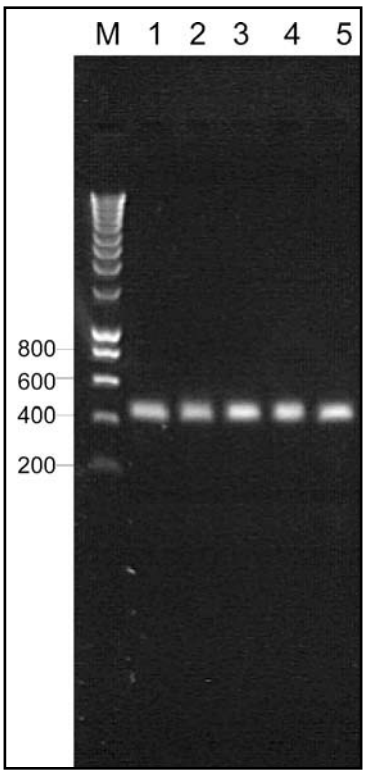

Figure 1. Expression of survivin in pituitary tumors. Electrophoretic separation showing representative RT-PCR results performed for pituitary tumors. A 424 bp fragment of survivin was amplified for pituitary tumors (lanes 1 – 4) and normal pituitary (lane 5) samples. Molecular size marker is given in lane M.

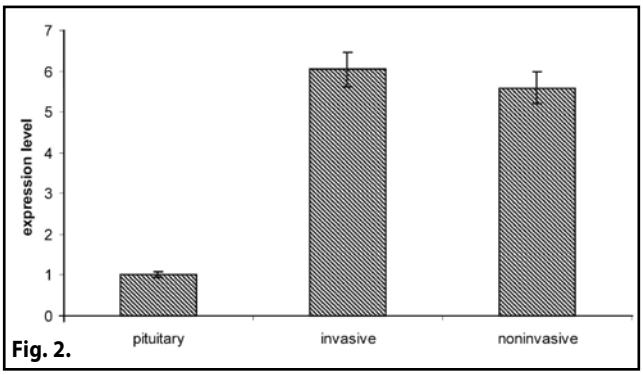

Figure 2: Relative expression level of survivin in pituitary and pituitary tumors. Total RNA was isolated from the tissues, and cDNA was synthesized. Pituitary tumors expressed increased survivin message relative to control normal pituitary tissue. Columns – mean of triplicate experiments; bars – SE

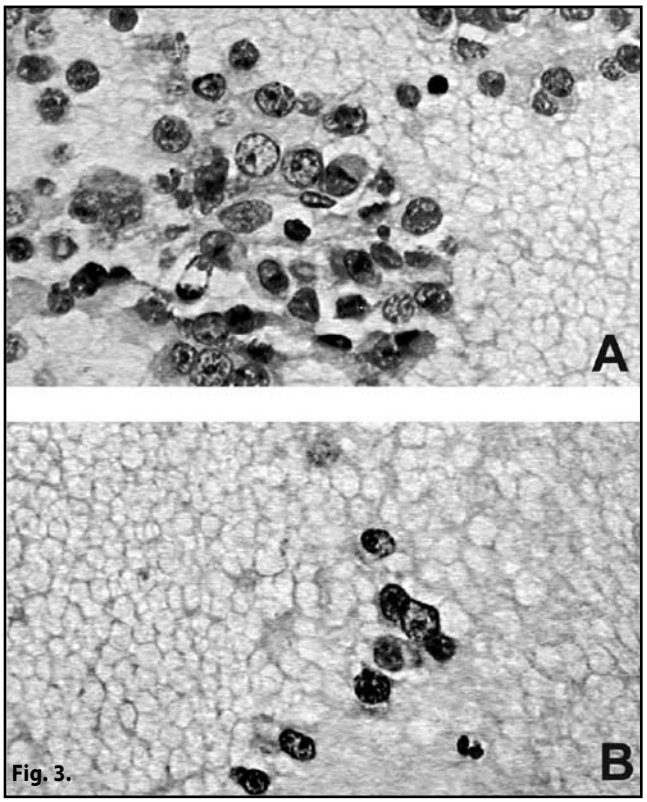

Figure 3: Immunolocalisation of survivin in pituitary tumors
A – Immunohistochemistry was performed using specific antibodies against survivin on paraffin section of tumor tissue. Hormone staining was localized predominantly in the nucleus of cells collected in clusters.
B – Positive immunostaining was also detected in single cells in normal pituitary tissue. Original magnification, x400

Immunostaining of tumor tissue was not uniform. Survivin was localized predominantly in cells collected in clusters, characterised by heterogeneous nuclear staining (Figure 3A). The presence of the protein in normal pituitary was also detected, however positive staining was restricted to small population of cells (Figure 3B).

No labeling was observed in the control reactions – where the primary antibodies were omitted (data not shown).

DISCUSSION

Survivin is a multifunctional protein that inhibits apoptosis, regulates cell division, and enhances angiogenesis [19]. It has been shown that its gene is ubiquitously expressed in embryonic and foetal organs as well in various types of tumors but becomes undetectable in most normal terminally differentiated tissues [20]. Thus, intense research has been focused on the potential role of survivin as a tumor biomarker.

Previous studies in benign brain and pituitary tumors demonstrated that survivin's gene is expressed in these tissues [16,18]. Our present findings showed that the presence of survivin is characteristic for pituitary tumors but also for normal pituitary tissue. However the level of the gene expression was 6-fold higher in tumors than in normal pituitary. This might indicate that the increase of survivin expression is one of the factors of pituitary neoplastic transformation. In 23 pituitary tumors examined by us both survivin mRNA and protein were detected. The immunohistochemical analyses confirmed the data of quantitative RT-PCR. This study showed that expression of survivin in normal pituitary tissue was restricted to small population of cells compared with neoplastic tissue where the large group of cells producing the protein were observed. Nuclear survivin observed in analysed tissues in contrast to cytoplasmic fraction revealed in other studies have been associated with unfavorable prognosis [21]. There are also some reports showing the association with favorable prognosis [22]. Nevertheless the fact that survivin expression is associated with the apoptosis inhibition and promotion of proliferation, there is rationale that increased survivin level would be associated with an aggressive tumor phenotype [19,23].

Since the interpretation of survivin localization (nuclear versus cytoplasmic) is a challenge even for experienced pathologists, it seems reasonable to rather measure the gene expression level and to use the amount of transcript for diagnosis and therapeutic decision.

Pituitary tumors are mostly benign adenomas that grow slowly, and in most of cases hormone products that are oversecreted lead to disturbed endocrine functions. Still, the tendency of the pituitary tumor to recur and infiltrate the surrounding tissues make the study of pituitary tumorogenesis an important problem.

The results of our study demonstrated that relative survivin mRNA expression in invasive and non inva-

sive tumors did not differ significantly, therefore it can not be correlated with the tumor type, the histological grade and the clinical outcome of patients. Unfortunately the experimental procedure used to establish the levels of survivin expression did not get the possibility to verify the number of neoplastic cells present in analyzed tissues. Thus, we can not conclude total survivin levels in particular tumors precisely. Further analysis of survivin expression profiles should show if survivin could be used as a prognostic marker for diagnosis and therapy of pituitary tumors, especially that recent reports demonstrated that survivin expressed in various types of tumors might be a target of gene silencing or serve as a potent immunogenic cancer vaccine [24,25].

CONCLUDING REMARKS

The present data showed overexpression of survivin in pituitary tumors. The levels of survivin expression in neoplastic tissue is about 6-fold higher than in normal pituitary. Further analysis should show if survivin could be used as a prognostic marker for diagnosis and treatment of pituitary tumors.

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