Survivin DEx3 as a biomarker of thyroid cancers: A study at the mRNA and protein level

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Abstract. Survivin and its splice variants DEx3 and 2B are involved in pathogenesis of numerous types of cancer. Proliferating cell nuclear antigen (PCNA) level correlates with cellular proliferation. The present study aimed to analyze the potential utility of survivin and its splice variants DEx3 and 2B as biomarkers for thyroid cancer. PCNA, survivin and its splice variants DEx3 and 2B expressions were analyzed in 22 tissue samples (15 thyroid cancers and 7 benign lesions) by reverse transcription-quantitative polymerase chain reaction and immunohistochemistry (IHC). There was significantly higher staining for survivin (P=0.019), survivin DEx3 (P=0.001), survivin 2B (P=0.0149) and PCNA (P=0.0237) in thyroid malignant tumors when compared with benign lesions. The receiver operating characteristics curve analysis has shown that the cut-off points of survivin IHC expression >2 [sensitivity 46.7%; specificity 100%; area under curve (AUC) 0.810; P=0.0005] and survivin DEx3 IHC expression >0 (sensitivity 86.7%; specificity 100%; AUC 0.933; P<0.0001) were the best predictors of thyroid malignancy. Additionally, PCNA staining >1 (sensitivity 93.3%; specificity 71.4%; AUC 0.790; P=0.0243) and survivin 2B >2 (sensitivity 46.7%; specificity 100%; AUC 0.824; P=0.0002) were the best predictors of thyroid cancer. In conclusion, the present study exhibited that survivin DEx3 expression has high specificity and sensitivity for discrimination between benign thyroid lesions and cancers. Survivin DEx3 may be considered a biological marker of thyroid malignancy and therefore applied in clinical practice.

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Introduction

The incidence of thyroid cancer in developed countries has increased (1). Histopathological examination remains the gold standard for the diagnosis and classification of thyroid lesions excised during surgery (2). In addition, molecular markers of thyroid cancers may be used as diagnostic and even prognostic tools (3,4). Certain markers may also be targets for non-conventional therapy.

Survivin, a protein belonging to the family of apoptosis inhibitors, is involved in cell-cycle regulation and proliferation (5,6). Survivin overexpression has been observed in types of endocrine cancer including differentiated and medullary thyroid cancer, while was not upregulated in benign pituitary adenoma (7-16). However, survivin has not been proven to discriminate between thyroid follicular cancer and adenoma (17,18). The two survivin splice variants 2B and DEx3 demonstrate different biological properties. Survivin 2B is involved in apoptosis activation. By contrast, anti-apoptotic survivin DEx3 is suggested to be associated with tumor aggressiveness, advanced stage and poor prognosis in numerous types of cancer (5). We have previously reported survivin and its DEx3 mRNA variant overexpression in thyroid cancer (19,20).

Another nuclear protein, proliferating cell nuclear antigen (PCNA), is also involved in replication, so its level correlates with cellular proliferation (21).

The aims of the present study were to confirm the findings on the overexpression of survivin and its DEx3 variant in thyroid cancer at the protein level using immunohistochemical (IHC) staining, and to evaluate the role of survivin and its splice variants DEx3 and 2B as potential biomarkers of thyroid malignancy. The present study additionally estimated the PCNA protein expression reflecting proliferative activity of tumors to better understand survivin and associated splice variant expression.

Materials and methods

Thyroid tissues. The present study group consisted of 15 thyroid cancer tissue samples: 5 from papillary thyroid

cancers; 5 from medullary thyroid cancers; 4 from follicular thyroid cancers; and 1 undifferentiated thyroid cancer. In total, 7 thyroid tissue samples derived from benign lesions served as controls: 4 cases of colloid nodules and 3 cases of follicular adenomas. The thyroid tissues were obtained from patients who had undergone thyroidectomy due to several medical reasons. The resected thyroid tissue specimens were divided into 2 samples. The first was immediately submerged in an RNA protective medium and stored at -80˚C awaiting reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The second sample was placed in a tissue processor, dehydrated and fixed in 10% neutral buffered formalin and embedded in paraffin for subsequent pathological examination and IHC staining. The present study was approved by the Ethics Committee of Poznań University of Medical Sciences (Poznań, Poland), and written informed consent was obtained from each patient.

RNA isolation and RT. With the use of 3-Zone RNA Isolation Reagent (Novazym Polska s.c., Poznań, Poland) total cellular RNA was extracted from all tissue samples including controls, according to the manufacturer's protocol (the procedure was conducted twice for each sample). Prior to the second 3-Zone purifying step, the RNA was suspended in 40 μl RNase-, DNase- and pyrogen-free H_2O, and 5 μl DNase and DNA digestion buffer (Zymo Research Corp., Irvine, CA, USA) was added. The reaction mix was incubated at 25˚C for 15 min. Subsequent to the concentration and purity measuring processes (NanoDrop ND-1000 spectrophotometer; Thermo Fisher Scientific, Inc., Waltham, MA, USA), agarose gel electrophoresis was performed in order to verify RNA integrity. Isolated RNA was reverse transcribed or stored at -80˚C until additional analysis. The cDNA synthesis was carried out as previously described using 1 μg of the RNA and universal oligo(d)T_{10} primer (Genomed, Warszawa, Poland) in the final volume reaction of 20 μl (22). Complementary DNA was synthetized, according to the reverse transcriptase manufacturer's protocol, using 50 ng/μl total RNA, 5 pm/μl universal oligo(d)T_{10} primer, 10 U/μl Expand Reverse Transcriptase, 1X Expand Reverse Transcriptase Buffer, 10 U/μl RNasin (RNase inhibitor) and 1 pm/μl dNTPs (all Roche Molecular Diagnostics, Pleasanton, CA, USA). The mixture of RNA, oligo(d)T_{10} primer and water was incubated 10 min at 65˚C. Subsequently the samples were incubated on ice. The thermocycling conditions were as follows: 10 min at 25˚C; 30 min at 55˚C; and 5 min at 85˚C.The cDNA was immediately used for the qPCR analysis.

qPCR analysis. To establish the expression levels of survivin (NCBI no., NM_001168), and its splice variants: *BIRC5*-DEx3 (NCBI no., NM_001012270.1) and BIRC5-2B (NCBI no., NM_001012271.1) TaqManÒ hydrolysis probe no. 36 (cat. no., 04687949001; Roche Applied Science, Manheim, Germany) and gene specific primers sets were used. The generated amplicon lengths and primers were: 88 bp for survivin sense, 5'-GCCCAGTGTTTCTTCTGCTT-3' and antisense, 5'-AACCGGACGAATGCTTTTTA-3'; BIRC5-2B sense, 5'-TCTGCTTCAAGGAGCTGGA-3' and antisense, 5'-AAAGTGCTGGTATTACAGGCGTA-3'; and 77 bp primers for *BIRC5*-DEx3 were sense, 3'-CAGTGTTTCTTCTGCTTCAAGG-5' and antisense, 3'-CTTATTGTTGGTTTCCTTTGC-5'. To prevent product synthesis from genomic DNA, the primer sets were designed to genic region with intragenic fragments between 1,874 and 8,670 bp in length, depending on the survivin variant. The assays were designed *in silico* using ProbeFinder Software (version 2.50; Roche Applied Science) and applied in qPCR reactions. Human HPRT Gene Assay (cat. no., 05 046 157 001; Roche Applied Science) served as reference reaction. qPCR was performed as previously described in a total reaction volume of 20 μl in a LightCycler® 2.0 carousel based system (Roche Applied Science) (20). All reactions were made in triplicates with the hot start 1X LightCycler® TaqMan® Master mix (Roche Applied Science). The probes and primers concentration were set to 200 nM. The thermal profile was as follows: Pre-incubation step, 95˚C, 10 min; 45 quantitation cycles (denaturation 95˚C, 10 sec; annealing/elongation 60˚C, 30 sec; fluorescence data acquisition 72˚C, 1 sec); and cooling step to 40˚C.

Reaction efficiency was calculated by comparing row data with the genes corresponding standard curve generated as described previously (22). Each reaction set included the negative no template control (total cellular RNA pool from different samples and isolation in RT reaction lack of transcriptase). As no contamination had been observed, the u*racil*-DNA glycosylase incubation step was omitted.

The qPCR raw data were collected and analyzed with the use of the LightCycler® Data Analysis (LCDA) Software (version 4.0.5.415; Roche Applied Science). Each sample was analyzed in a triplicate and the average threshold value Cq was calculated. The $2^{-\Delta\Delta Cq}$ method was used (23). Comparing the data with standard curve and reference genes fluorescence data acquisition, the concentrations ratios (Cr) were calculated for each patient and control sample.

IHC staining. Formalin fixed paraffin-embedded tissue specimens of thyroid cancers and benign lesions were cut in 5 μm thick sections. IHC reactions, subsequent to non-specific antigen binding in blocking solution, were performed with the use of rabbit monoclonal anti-survivin antibody (dilution, 1:250; cat. no., ab76424; Abcam, Cambridge, UK), rabbit polyclonal anti-survivin DEx3 antibody (dilution, 1:250; cat. no., ab3731; Abcam), rabbit polyclonal anti-survivin 2B antibody (dilution, 1:250; cat. no., ab3729; Abcam) and rabbit monoclonal anti-PCNA antibody (dilution, 1:250; cat. no., ab92552; Abcam). Reactions were visualized through subsequent incubation with secondary biotinylated goat anti-rabbit immunoglobulin antibody (1:100; cat. no. E0432; Dako, Glostrup, Denmark), streptavidin-horseradish peroxidase complex (LSAB2-HRP anti-rabbit/mouse; cat. no. K0675; DAKO, Glostrup, Denmark) and 3,3'-diaminobenzidine chromogen. The assessment was performed using light microscopy via the Olympus CX41 microscope (Olympus Corporation, Tokyo, Japan). The intensity of survivin/survivin 2B/survivin DEx3 expressions was evaluated using the modified semi-quantitative immunoreactive score (IRS) approach, according to Remmele and Stegner (24). The percentage of positively stained cells (PP) is scored (range, 0-4 points) and the staining intensity (SI) is scored (range, 0-3 points), and these scores are combined (IRS=SI x PP). The final score was presented on a five-point scale (range, 0-4 points): 1-2 IRS points=1 point; 3-4

IRS points=2 points; 6-8 IRS points=3 points; and 9-12 IRS points, 4 points.

Statistical analysis. Statistical analyses were performed using MedCalc for Windows, version 15.8 (MedCalc Software, Ostend, Belgium). P<0.05 was considered to indicate a statistically significant difference. A comparison of the analyzed parameters between 2 groups was performed using the Mann-Whitney U test. The strength of the association between analyzed parameters was measured with Spearman's rank correlation coefficient test. Receiver operating characteristics (ROC) curves were calculated to determine the potential of analyzed parameters in order to discriminate between thyroid cancer and benign lesions. An optimal cut-off point was calculated according to the highest accuracy (minimal false negative and false positive results). The area under the ROC curve (AUC) was used to check the prognostic value of particular parameters.

Results

Patients and IHC staining. The median age of patients with thyroid cancer was 59 years [interquartile range (IQR) 41.5-67.5 years] and in patients with benign lesions was 52 years (IQR 35-54.5 years; P=0.0668). Clinical data, including patient demographics, tumor histology and staging, are presented in Table I. A positive staining of survivin and survivin 2B was found in 92.9% of the thyroid cancers samples and in 57.1% of controls. Survivin DEx3 was recognized in 85.7% of the thyroid cancer samples and in none of benign specimens. When PCNA expression was analyzed in the same group, positive staining was found in 100% of thyroid cancer specimens and in 57.1% of benign lesions. There was significantly higher staining for survivin (P=0.019), survivin DEx3 (P=0.001), survivin 2B (P=0.0149) and PCNA (P=0.0237) in thyroid malignant tumors when compared with benign lesions.

Relationship between IHC and mRNA expressions. There was a strong correlation between survivin DEx3 mRNA and IHC expressions (P=0.0014; r=0.639). There was also a trend for association between survivin mRNA overexpression and IHC upregulation (P=0.05; r=0.421). The present study has not observed the correlation between survivin 2B mRNA and protein expression (P=0.2149). A significant association between survivin DEx3 and IHC expressions of survivin (P=0.0007; r=0.670), survivin 2B (P<0.0001; r=0.763), and PCNA (P=0.0024; r=0.614) were also observed (Fig. 1). There were no correlations between tumor stage and survivin DEx3 mRNA and protein expressions.

IHC expression in the prognosis of thyroid malignancy. The ROC curve analysis has shown that the cut-off point of survivin IHC expression >2 (sensitivity 46.7%; specificity 100%; AUC 0.810; P=0.0005) and survivin DEx3 IHC expression >0 (sensitivity 86.7%; specificity 100%; AUC 0.933; P<0.0001) were the best predictors of thyroid malignancy (Fig. 2). PCNA staining >1 (sensitivity 93.3%; specificity 71.4%; AUC 0.790; P=0.0243) and survivin 2B >2 (sensitivity 46.7%; specificity 100%; AUC 0.824; P=0.0002) were the best predictors of thyroid cancers.

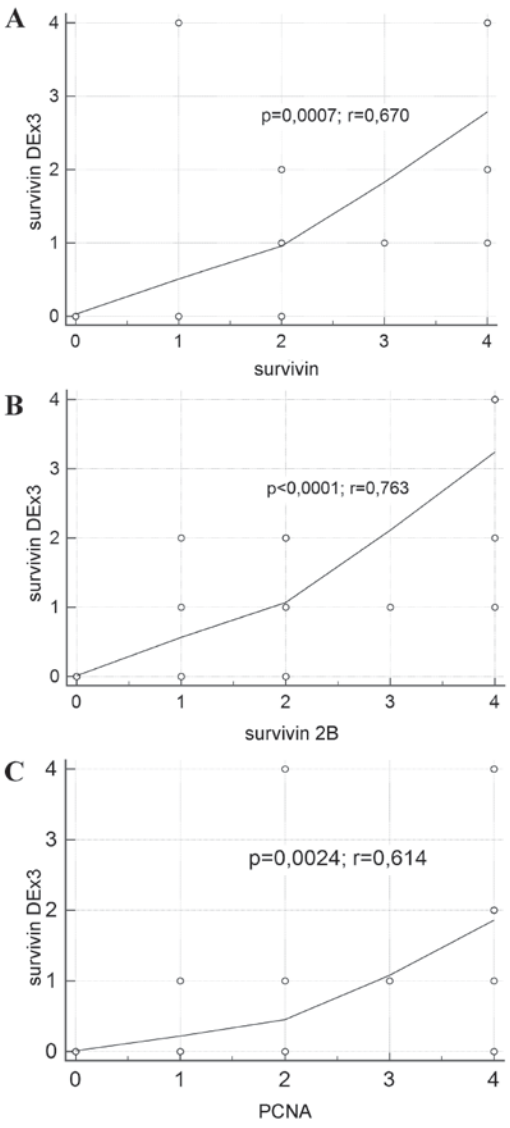

Figure 1. Positive association between survivin DEx3, and (A) survivin, (B) survivin 2B and (C) PCNA immunohistochemical expressions in benign and malignant thyroid lesions. The immunoreactive scores of the proteins were compared. PCNA, proliferating cell nuclear antigen.

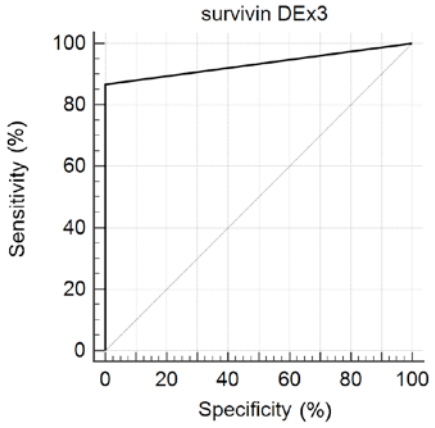

Figure 2. Survivin DEx3 immunohistochemical expression in the prognosis of thyroid malignancy. Survivin DEx3 IHC expression >0 was the best predictor of thyroid malignancy (sensitivity, 86.7%; specificity, 100%; area under the curve, 0.933; P<0.0001).

Table I. Clinicopathological data of patients from the study and control groups.

	Patients, n	Age, years	Gender	Histology, n	Staging, n
Thyroid cancer	15	Me 59	F 13	PTC 5	T1- 4
		(IQR 41.5-67.5)	M 2	FTC 4	T2- 3
				MTC 5	T3- 4
				UTC 1	T4- 4
Benign lesions	7	Me 52	F 4	Colloid nodule 4	-
		(IQR 35-54.5)	M 3	Follicular adenoma 3	

Me, median; T, tumor stage; IQR, interquartile range; F, female; M, male; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; MTC, medullary thyroid cancer; UTC, undifferentiated thyroid cancer.

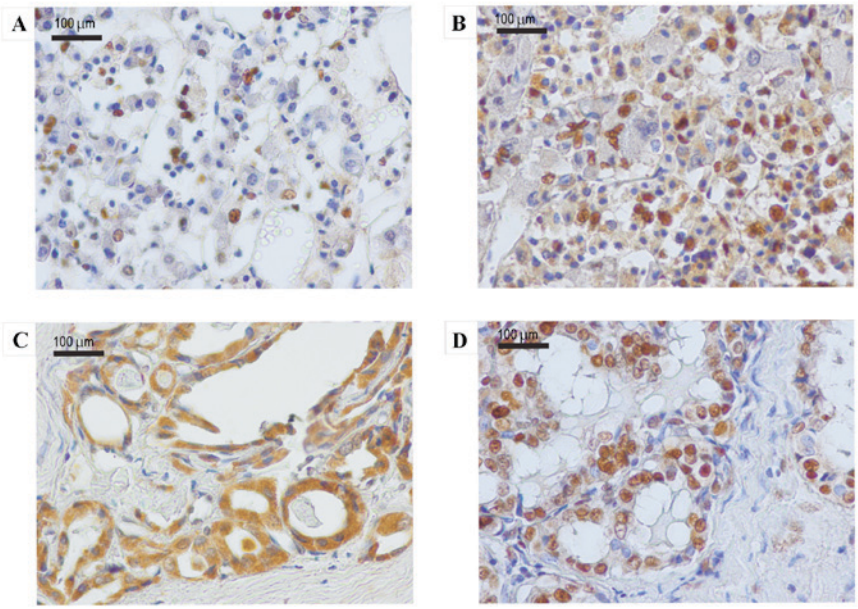

Figure 3. Immunohistochemical nuclear and cytoplasmic expression of (A) survivin, (B) survivin DEx3, (C) survivin 2B (D) and PCNA in cancer cells of follicular thyroid cancer. PCNA, proliferating cell nuclear antigen.

Fig. 3 shows cytoplasmic and nuclear staining of survivin, its splice variants DEx3 and 2B, and PCNA in thyroid cancer.

Discussion

In the present study, it was shown that survivin DEx3 expression has high specificity and sensitivity for discrimination between benign thyroid lesions and cancer. As a result, survivin DEx3 may be considered a biological marker of thyroid malignancy and therefore applied in clinical practice. To the best of our knowledge, this is the first study evaluating survivin DEx3 IHC expression in thyroid lesions. These findings are in agreement with our previous studies showing overexpression of survivin DEx3 *mRNA* in thyroid cancer (19,20). Using RT-qPCR, the present study also observed a positive association between the overexpression of survivin DEx3 mRNA and tumor aggressiveness. However, due to the limited sample size in the present study, the association between survivin DEx3 IHC overexpression and tumor stage and metastases is not confirmed.

Survivin protein overexpression in thyroid cancers has been previously reported (25). Pannone *et al* (26) observed enhanced cytoplasmic localization of survivin in differentiated thyroid cancer, whereas poorly differentiated and anaplastic thyroid cancer exhibited predominant nuclear protein staining by IHC. The authors hypothesized that this fact may be explained by the nuclear location of survivin splice variant DEx3. Recently, Selemetjev *et al* (27) reported stronger IHC staining of survivin in anaplastic in comparison with papillary thyroid cancer. Based on this observation, the authors suggested that survivin may perform a role in the progression of thyroid malignancy.

The present study also exhibited strong positive correlation between survivin DEx3 and PCNA expressions at the protein level in thyroid lesions. Upregulation of PCNA in thyroid cancer has been previously observed (28,29). In comparison with PCNA staining the current study observed that survivin DEx3 expression exhibited higher specificity with similar sensitivity. Survivin DEx3 showed stronger staining compared with survivin and survivin 2B. These findings

also support the potential clinical utility of survivin DEx3 upregulation in discrimination between malignant and benign types of thyroid tumor. This issue is particularly important among patients with thyroid nodules in whom the decision about thyroidectomy has to be made. Molecular biomarkers with high specificity and sensitivity could be helpful in the decision-making process, and certain patients may avoid unnecessary invasive procedures. Long-term observations may identify whether survivin DEx3 overexpression in thyroid cancer may be helpful in identifying patients with poor prognosis unresponsive to conventional therapy. Targeting of survivin gene may be a novel therapy in patients who did not respond to conventional treatment (30).

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