Arch. Biol. Sci., Belgrade, 63 (4), 955-963, 2011 DOI:10.2298/ABS1104955S

SURVIVIN IN RELATION TO BCL-2, BAX AND *IN SITU* APOPTOTIC CELL DEATH IN ANAPLASTIC THYROID CARCINOMA

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Abstract -Anaplastic thyroid carcinoma (ATC) is a rare but highly aggressive human malignancy. It is known that disturbances in apoptotic pathways have a great impact on tumor progression and aggressiveness. In this study the apoptosis-related molecules Bcl-2 (antiapoptotic), Bax (proapoptotic) and survivin (an inhibitor of apoptosis) were analyzed immunohistochemically in thirty archival cases of ATC. *In situ* apoptotic cell death was analyzed by the TUNEL method. Mean Bcl-2 staining score (calculated from individual scores from 0-3) was low compared to those for Bax and survivin ($p<0.05$). High expression of survivin was associated with high Bax expression, and was significantly segregated from high Bcl-2 expressing cases ($p<0.05$). Despite high Bax expression, apoptotic cell death was low in the investigated carcinomas. In addition, the mean apoptotic index in high survivin expressing carcinomas was significantly lower than in low survivin expressing carcinomas ($p<0.05$). It could be concluded that down-regulation of Bcl-2 is counterbalanced by up-regulation of survivin, which may overcome the effects of high Bax expression, and, at least partly, explain the low apoptosis rate and high biological aggressiveness of ATC.

Key words: Anaplastic thyroid carcinoma, apoptosis, Bax, Bcl-2, survivin

UDC 616.441-006.6

INTRODUCTION

Malignant tumors of the thyroid gland are classified as papillary, follicular, medullary and anaplastic. Carcinomas originating from the follicular epithelium are generally divided into two groups: well-differentiated (papillary or follicular) carcinomas and undifferentiated (anaplastic) carcinoma, while medullary carcinoma originates from parafollicular thyroid cells.

Contrary to well-differentiated carcinomas, which in general have a favorable prognosis, anaplastic thyroid carcinoma (ATC) is one of the most malignant and aggressive types of human tumors. The majority of patients diagnosed with ATC die within a few months after diagnosis due to suffocation as a consequence of locoregional disease extension or overwhelming distant metastases (Aldinger et al., 1978; Giuffrida et al., 2000; O' Neill et al., 2005; Wiseman et al., 2007).

Great clinical efforts have been made to control the disease locally, since it is usually very aggressive and invasive when diagnosed. In addition, molecular and genetic changes underlying the progression

of malignancy of thyroid tumors have been the subject of numerous investigations (Smallridge et al., 2009).

It is well-established that two processes, proliferation and apoptosis (programmed cell death), have a great impact on tumor progression and aggressiveness. Apoptosis is a form of physiological cell death driven by an intrinsic cellular suicide program aimed at excluding unwanted, infected or transformed cells without inflammation. The mechanism involved in the regulation of cell death is not yet completely understood, but members of the Bcl-2 family of proteins and their activators and inhibitors, seem to be the key regulators of this process (Burlacu, 2003; Kirkin et al., 2004). Bcl-2 protein is an antiapoptotic molecule that maintains the integrity of the mitochondrial membrane by preventing cytochrome c release from the mitochondria, which is needed for the proteolytic degradation of the cell by caspases (proteolytic enzymes) which are the final executors of programmed cell death. Unlike Bcl-2, its homologue Bax, a cytosolic protein, allows disruption of the mitochondrial membranes and caspases activation. The Bax gene (transcriptionally activated by p53, a tumor suppressor protein), was established as an inhibitory regulator of cell proliferation (Levine et al., 1991). If unsuccessful, p53 can also eliminate cells through the Bcl-2/Bax apoptotic pathway. The ratio between Bcl-2 and Bax appears to be important in deciding cell life or death (Reed, 1994).

Abnormalities in the control of programmed cell death play an important role in tumorigenesis. The expression of apoptosis-related molecules in thyroid carcinoma has been widely investigated, although with some controversial reports. The low rate of apoptotic cell death in thyroid carcinomas, and especially in ATC, despite the presence of the proapoptotic molecule Bax and tumor suppressor-p53, is still not understood (Manetto et al., 1997; Moore et al., 1998; Yoshida et al., 1999; Sreelekha et al., 2000; Farid et al., 2001; Saltman et al., 2006; Cvejic et al., 2009).

A candidate molecule influencing the apoptotic balance in cancer was identified as survivin (Ambrosini et al., 1997), a member of the inhibitor of the apoptosis protein (IAP) family (Deveraux and Reed, 1999). Survivin is a multifunctional protein that suppresses apoptosis by inhibiting caspase-3 and -7 activities (Tamm et al., 1998) and regulates the cell cycle in the G2/M phase (Li et al., 1998; Altieri, 2001). Unique among other IAP proteins, survivin is expressed during embryonic and fetal development. It is undetectable in normal adult tissues, but it is prominently overexpressed in the most common human cancers, including breast, gastric and colorectal carcinomas and lymphomas (Ambrosini et al., 1997; Duffy et al., 2007).

Thus, to gain a better insight into the molecular disturbances which underlie the biological behavior of ATC, we examined the expression of the apoptosis related molecules: Bcl-2 (antiapoptotic), Bax (proapoptotic), survivin (apoptosis inhibitory protein) and furthermore analyzed *in situ* apoptotic cell death in these tumors.

MATERIAL AND METHODS

Tissue samples

Thirty formalin-fixed, paraffin-embedded tissues of anaplastic thyroid carcinoma were obtained from the archival material of the Institute of Endocrinology, Diabetes and Diseases of Metabolism, KCS, Belgrade. Histological slides from the thyroid tumor tissue stained with hematoxylin and eosin were reevaluated by the pathologist. Cytological features (large polygonal or spindle-shaped cells, frequently multinucleated and containing abnormal mitotic figures) confirmed the diagnosis of ATC.

Immunohistochemistry

Immunohistochemistry was performed using monoclonal antibodies against human Bcl-2 (clone Bcl 2-100, Sigma, Germany) and survivin (clone SPM331, Abcam plc, Cambridge, UK), and rabbit

polyclonal antibody against Bax (Dako, Carpinteria, California, USA). Positive reactions were identified using a streptavidin-biotin-peroxidase detection system (Vector Laboratories, Burlingame, California, USA). Tissue sections (4-6 μm thick) from each block were deparaffinized with xylene and rehydrated through a series of descending concentrations of ethanol. Endogenous peroxidase activity was blocked with 0.3% H_2O_2/methanol for 30 min followed by incubation with non-immune horse serum for 20 min to block non-specific binding. Tissue sections were then incubated with primary antibody against Bcl-2 (1:200 dilution), primary antibody against Bax (1:25 dilution) or primary antibody against survivin (1:250) at 4˚C overnight. This was followed by incubation with biotinylated horse anti-mouse IgG (for Bcl-2 and survivin) or with biotinylated goat-anti rabbit IgG (for Bax) for 30 min and thereafter with the avidin-biotin-peroxidase complex for 30 min. Between each step, sections were washed three times in phosphate buffered saline (PBS). The reaction was visualized using 3, 3'-diaminobenzidine tetra hydrochloride (DAB) solution as a chromogen. After counterstaining with hematoxylin the slides were dehydrated in ascending ethanol, cleared with xylene, mounted with coverslips using a permanent mounting medium and thereafter examined using a Reichert-Jung microscope supplied with a Photostar automatic camera system. Controls were incubated with PBS in place of the primary antibody and no positive staining was observed.

Scoring of immunohistochemical staining

Cytoplasmic staining for Bcl-2, Bax and survivin was scored by two independent observers as follows: (-, 0) no staining; (-/+, 1) weak or focal staining; (+, 2) moderate staining in the majority of cells and (++, 3) strong staining in the majority of cells. The mean staining scores for Bcl-2, Bax and survivin were calculated (mean ± SD) from the individual staining score for each case (0-3) in the group. For ease of comparison, Bcl-2, Bax and survivin expressions were dichotomized into low expression (0-1) versus high expression (2-3).

In situ detection of apoptotic cells

Apoptotic cells in the tissue sections were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling technique (TUNEL), using a commercial apoptosis kit (TACS ™ TdT Kit, R&D Systems Inc., Minneapolis, USA), according to the supplier's instructions. In brief, the tissue sections of 30 cases of ATC were deparaffinized with xylene and ethanol and rinsed with PBS. The sections were then treated with proteinase K in PBS, followed by quenching of endogenous peroxidase. Biotinylated dNTP mix was added to the 3'-OH ends of DNA by terminal deoxynucleotidyl transferase (TdT). After incubating with streptavidin-horseradish peroxidase, the sections were stained with diaminobenzidine (DAB) and counterstained with hematoxylin. Finally, the sections were dehydrated in ethanol, cleared with xylene and mounted with coverslips in a permanent medium. Experimental controls included during the performance of the TUNEL method protocol were: TACS-nuclease-treated thyroid tissue sections as a positive control and the omission of the TdT reaction step as a negative control, according to the supplier's instructions.

The percentage of apoptotic cells was determined by light microscope examination. The mean apoptotic score was calculated (mean ± SD) from the individual staining scores for each case in the group, and assigned as an Apoptotic Index (AI).

Ethical issues

The study protocol was approved by the Ethics Committee at the Clinical Center of Serbia, Belgrade, which conforms to the provisions of the Declaration of Helsinki.

Statistical analysis

Statistical comparisons of data were made using χ^2 test, Fisher's exact test or the Mann-Whitney U-test, as indicated in the Results section. A value $p<0.05$ was considered to be statistically significant.

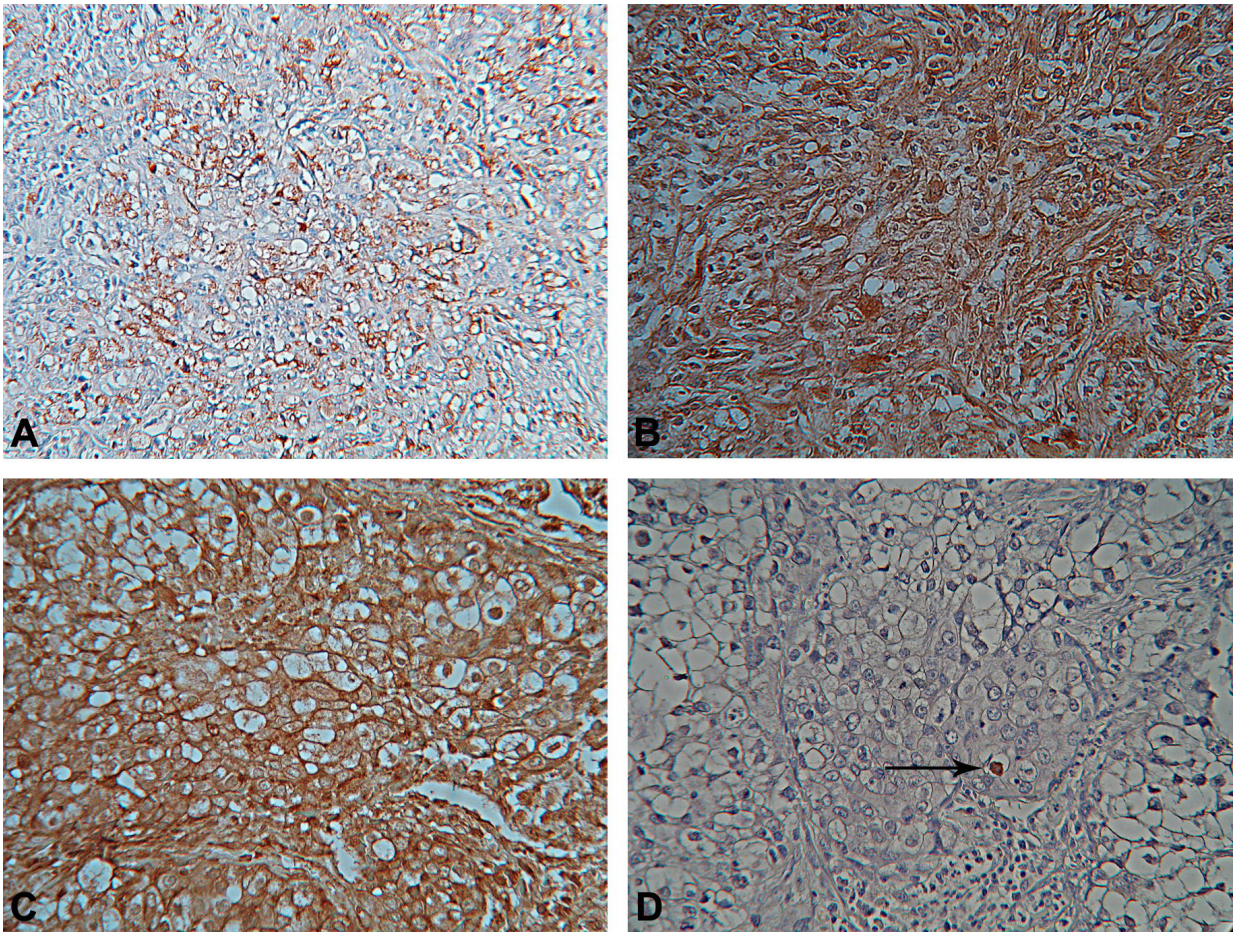

Fig.1. Immunohistochemical expression of Bcl-2, Bax and survivin and *in situ* detection of apoptotic cells in anaplastic thyroid carcinoma**. (A)** Low cytoplasmic positivity for Bcl-2. **(B)** High Bax cytoplasmic immunopositivity. **(C)** High immunohistochemical expression of survivin. **(D)** *In situ* labeling for apoptosis in the corresponding area of (C) detected only one apoptotic tumor cell (arrow).
ABC technique, hematoxylin diaminobenzidine: A, B, C. TUNEL technique, hematoxylin diaminobenzidine: D. Original magnifications: A x20; B,C,D x40.

RESULTS

Our immunohistochemical staining results are presented in Tables 1-3 and representative microphotographs are shown in Fig. 1 (A-D).

Immunohistochemical expression of Bcl-2 protein was visualized as cytoplasmic staining and found in 24 of the 30 ATC cases analyzed, but mostly as weak or focal staining (Table 1, Fig. 1-A). There were only 8 cases (26.6%) which exhibited moderate expression of Bcl-2 (scores 2), which indicates scarcity of this antiapoptotic protein in anaplastic thyroid cancer cells.

Bax protein was expressed in 29 of the 30 cases of ATC (Fig. 1-B), with strong staining intensity in most of them, i.e. 24 (80.0%) cases were in the high Bax expressing group (scores 2 or 3).

Immunohistochemical staining for survivin revealed cytoplasmic positivity of cancer cells in 27 of the 30 cases (90.0%). Occasionally, some nuclei were also found to be positive. The distribution of

Table 1. Immunohistochemical expression of Bcl-2, Bax and survivin in anaplastic thyroid carcinoma (ATC).

Staining score	Bcl-2	Bax	survivin
- (0)	6	1	3
-/+ (1)	16	5	6
+ (2)	8	10	4
++ (3)	0	14	17
Mean score	1.067 ± 0.691[a]	2.233 ± 0.858[b]	2.462 ± 1.085[c]

Staining score: (-,0) no staining; (-/+,1) weak or focal staining; (+,2) moderate staining in the majority of cells, (++,3) strong staining in the majority of cells.
Mean score: as detailed in Material and Methods section.
Statistically significant differences ($p<0.05$) for a versus b and a versus c (Mann-Whitney U-test).

Table 2. Correlation between immunohistochemical expression of survivin and Bcl-2 or Bax expression in anaplastic thyroid carcinoma (ATC).

	Survivin high expressing cases (n=21)
Bcl-2 low expressing cases	16/21 (76.2 %)
Bcl-2 high expressing cases	5/21 (23.8 %)[a]
Bax low expressing cases	4/21 (19.1 %)
Bax high expressing cases	17/21 (81.0 %)[b]

Low expressing cases: staining scores (-,0) and (-/+,1). High expressing cases: staining scores (+,2) and (++,3).
Statistically significant difference ($p<0.05$) for a versus b (Fisher's exact test).

Table 3. The relationship between survivin expression and apoptotic cell death in anaplastic thyroid carcinoma (ATC).

	n	AI (%)
Survivin low expressing cases	9	0.556 ± 0.527[a]
Survivin high expressing cases	21	0.143 ± 0.359[b]

AI: as detailed in Material and Methods section.
Statistically significant difference ($p<0.05$) for a versus b (Mann-Whitney U-test).

survivin immunohistochemical scores showed that 21 (70.0%) cases were in the high expressing group (Table 1, Fig. 1-C).

Since positive carcinomas showed variability in the immunostaining pattern of the analyzed proteins, the average staining scores for Bcl-2, Bax and survivin were calculated (mean ± SD) from the individual staining scores for each case (0-3). Thus, the average staining score for Bax and survivin (2.233 ± 0.858 and 2.462 ± 1.085, respectively) showed that these proteins were expressed at similar high levels in ATC, while the average staining score for Bcl-2 (1.067 ± 0.691) was significantly lower ($p<0.05$; Mann-Whitney U-test).

The relationship between survivin and Bcl-2 or Bax is presented in Table 2. High expression of survivin showed a strong positive correlation with the high expression of Bax, and it was significantly segregated from high Bcl-2-expressing cases (17/21, 81.0 % versus 5/21, 23.8 %; $p<0.05$; Fisher's exact test).

The number of apoptotic cells detected by *in situ* labeling was low, with zero up to ten positive cells per 10 HPFs in most of the carcinomas studied. The mean AI of the 30 ATC cases analyzed was 0.267 % (SD, 0.449%). The relationship between apoptotic cell death and survivin expression was examined and is presented in Table 3 and Fig. 1-C, D. The mean AI in high survivin expressing carcinomas was 0.143% (DS, 0.359%), which was significantly lower than the mean AI of 0.556% (SD, 0.527%) observed in low survivin expressing tumors ($p<0.05$; Mann-Whitney U-test).

DISCUSSION

Anaplastic thyroid carcinoma (ATC) is a rare and rapidly lethal human malignancy. The biological behavior of each tumor depends on a number of factors, including the balance between proliferation and programmed cell death (apoptosis). As a fast growing tumor, anaplastic thyroid carcinoma is characterized by a high proliferative capacity that is not counterbalanced by an appropriate rate of apoptotic cell death. Although the expression of the genes regulating apoptosis in thyroid malignant tissue has been extensively studied during the last years, the complexity of genetic defects in the apoptotic pathways in thyroid carcinomas are still not clearly understood. In this study we examined the expression of apoptosis-related molecules: Bcl-2 (antiapoptotic), Bax (proapoptotic), survivin (apoptosis inhibitory protein) and furthermore analyzed *in situ* apoptotic cell death in anaplastic thyroid carcinoma.

The Bcl family of proteins plays an important role in the regulation of apoptosis (Burlacu, 2003; Kirkin et al., 2004). Representative members of this family are Bcl-2 and Bax. With regards to thyroid tumors, there is general agreement that Bcl-2 is expressed at high levels in normal thyroid tissue as well as in well-differentiated thyroid carcinomas (Pilotti et al., 1994; Pollina et al., 1996; Moore et al.,1998; Mitselou et al., 2004; Aksoy et al., 2005; Cvejic et al., 2008). However, its expression was reported to decrease with dedifferentiation and aggressiveness (Pollina et al., 1996; Moore et al., 1998; Saltman et al., 2006; Cvejic et al., 2009). In this study we found low expression of Bcl-2 protein in the ATC cases analyzed, which is in agreement with previous reports.

The proapoptotic molecule Bax has been investigated to a lesser extent in thyroid carcinomas, with conflicting results, and its role in thyroid tumorigenesis and tumor biology is controversial. While some authors could not detect Bax in differentiated thyroid carcinomas (Manetto et al., 1997), others reported Bax expression in all or in the majority of differentiated carcinomas investigated (Hermann et al., 2001; Letsas et al., 2005). Considering Bax expression in ATC, Branet et al. (1996) reported that it was either not expressed or was only weakly expressed, while Manetto et al. (1997) found strong staining for Bax in ATC. The results of our previous (Cvejic et al., 2009) and current study revealed high cytoplasmic expression of Bax in almost all cases of ATC.

Considering the high Bax and low Bcl-2 expression levels demonstrated in ATC, and the fact that programmed cell death is regulated by a balance

between proapoptotic and antiapoptotic factors, it should be expected that this type of thyroid cancer exhibits an increased apoptotic death rate (Yoshida et al., 1999; Sreelekha et al., 2000). However, we have observed here that, in spite of high Bax and low Bcl-2 expression, there was a low apoptotic index in ATC. With regard to the complexity of the proapoptotic and antiapoptotic pathways, with multiple factors involved, down-regulation of only one antiapoptotic factor (i.e. Bcl-2) may not always result in enhancement of apoptotic cell death. Thus, other defects across the apoptotic pathway in the malignant cell, for example defects in the functions of downstream effectors of Bax, such as caspases, with their activators and inhibitors, should be considered.

In this study we investigated survivin expression in ATC for the first time along with analysis of Bcl-2 and Bax expression, and analyzed their relation to *in situ* apoptotic cell death.

Survivin is a recently identified inhibitor of apoptosis (IAP), which directly inhibits caspase-3 and-7 activities and regulates the cell cycle in the G2/M phase, being therefore a bifunctional protein that acts as a suppressor of apoptosis and plays a role in cell proliferation (Tamm et al., 1998; Li et al., 1998). Survivin has been found to be strongly expressed in many common human cancers (Duffy et al., 2007) and in some of them, for example in breast cancer, overexpression of this protein has been considered to be a poor prognostic factor (Tanaka et al., 2000).

There are only a few studies on survivin expression in thyroid carcinomas. Survivin protein was detected in well-differentiated thyroid carcinomas by Haghpanah et al. (2006), Antonaci et al. (2008) and Dong et al. (2006), but there are only few data on survivin immunohistochemical expression in ATC. Ito et al. (2003) found a higher incidence of survivin expression in ATC (84 %) than in papillary carcinoma (20 %) cases, suggesting that this protein is related to the dedifferentiation of thyroid carcinoma. Similarly, Zhang et al. (2009) reported that overexpression of survivin in ATC compared with well- differentiated carcinoma is an unfavorable prognostic factor.

Our results revealed survivin immunohistochemical expression in 90.0 % of the ATC cases examined. In addition, high expression of survivin showed a strong positive correlation with high Bax expression and reduced apoptosis rate. Thus, a characteristic of ATC is the high expression of survivin, accompanied by a high expression of Bax, low expression of Bcl-2 and low apoptotic cell death.

The existence of a low apoptosis and elevated survivin expression suggests an apoptosis inhibitory function for survivin in anaplastic thyroid carcinoma. While Bcl-2, as an antiapoptotic molecule, exerts its effect by maintaining mitochondrial membrane integrity and thereby prevents the apoptotic effects of Bax (Burlacu, 2003; Kirkin et al., 2004), survivin exerts its antiapoptotic function in Bax downstream pathways by targeting caspase-3 (Tamm et al., 1998). Thus, it seems that down-regulation of Bcl-2 is counterbalanced with an up-regulation of survivin, which may overcome the effects of high Bax expression, and, at least partly, explain the low apoptosis rate and high biological aggressiveness of ATC. In this context, survivin-targeted therapy might be promising for these patients in the future.

Acknowledgment - Supported by the Ministry of Education and Science of the Republic of Serbia, project 173050; "Molecular characterization of thyroid gland tumors: biological and clinical aspects."

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