

## Determination of apoptosis and cell cycle modulators (p16, p21, p27, p53, BCL-2, Bax, BCL-xL, and cyclin D1) in thyroid follicular carcinoma, follicular adenoma, and adenomatous nodules via a tissue microarray method

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**Background/aim:** To identify the role of gene products associated with apoptosis and cell cycle in the pathogenesis of thyroid follicular neoplasm.

**Materials and methods:** Thirty follicular adenomas (FAs), 16 follicular carcinomas (FCs), and 20 adenomatous nodules (ANs) were investigated with immunohistochemical staining of p16, p21, p27, p53, Bcl-2, Bax, Bcl-xL, and cyclin D1 via a tissue microarray method.

**Results:** Bcl-2 showed a significant difference between the benign groups (AN and FA) and the malignant group (FC). Bax was significantly higher in the FC group. p53 was lowest in the AN group and highest in the FC group with significant differences between the groups. p16 was significantly higher in the FC group than in the other groups. There was a significant difference between the AN group and neoplastic lesions in terms of p21 staining. The number of cases with positive p27 was lower in the AN group than the neoplastic groups. There was no significant difference in terms of Bcl-xL and cyclin D1.

**Conclusion:** Cell cycle modulators, led by the Bcl-2 family, played an important role in the pathogenesis of thyroid follicular neoplasm, and p53, p16, and p21 in particular played a role in the carcinogenesis of FC.

**Key words:** Thyroid, follicular neoplasm, p16, p21, p27, p53, Bcl-2, Bax, Bcl-XL, Cyclin D1, immunohistochemistry, tissue microarray

### 1. Introduction

Despite various studies on cell cycle modulators such as p16, p21, p27, p53, Bcl-2, Bax, Bcl-XL, and cyclin D1 in thyroid lesions, the current literature includes no large-scale study that evaluates all of these as a whole. The present study aimed to identify the role of these gene products, which are associated with apoptosis and cell cycle and are intercorrelated, in pathogenesis within a broad spectrum of samples of follicular carcinomas (FCs), follicular adenomas (FAs), and adenomatous nodules (ANs).

### 2. Materials and methods

#### 2.1. Cases

In the present study, 30 FA and 16 FC cases, which were diagnosed based on the thyroidectomy materials or the blocks sent for consultation between 2006 and 2010 in the pathology department of our university hospital,

were evaluated. Twenty cases of AN were included as the control group.

The diagnoses were verified by reexamining the preparations stained with hematoxylin & eosin (HE). The preparations and paraffin blocks that contained the higher amounts of tissue and represented the lesions were selected.

#### 2.2. Preparation of tissue microarray blocks and sections

In order to obtain suitable blocks for the tissue microarray (TMA) method, routine paraffin and wax (Merck Product No. 1115449020, Darmstadt, Germany) were mixed at a proportion of 1/10 (wax/paraffin) and melted at 64 °C. Blocks were obtained using a 3DHISTECH Ltd. Manuel TMA Kit (Budapest, Hungary). Using a TMA punch pencil, 2 mm of target tissues were taken from the paraffin blocks with marked lesion area and 16–18 tissue samples were embedded in each TMA block. One slide was

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prepared from each block for HE staining and 10 slides were prepared for immunohistochemical staining.

### 2.3. Immunohistochemical staining method

Immunohistochemical staining was performed in 4- $\mu$ m sections taken from TMA blocks onto the positive-load specific slides via a fully automatic device (Ventana Benchmark XT, Tucson, AZ, USA) and relevant immunohistochemical kit (DAB Substrate System, AEC Detection Kit, EZ Prep, LCS, SSC Solutions, RBS, Amplification, Protease, Block Solutions, Hematoxylin; Ventana).

For immunohistochemical staining, purified mouse antihuman p16 (INK4) Ab-4 (16P04, MS-887-P1, LabVision/NeoMarkers, Fremont, CA, USA; 1/25 dilution-40 min), mouse antihuman p21 Waf1 monoclonal antibody (Clone SPM306, E6330, Spring Bioscience, Pleasanton, CA, USA; 1/50 dilution-1 h), rabbit antihuman p27 Kip1 polyclonal antibody (E2600, Spring Bioscience; 1/100 dilution-12 min), rabbit antihuman p53 monoclonal antibody (Clone SP5, M3050, Spring Bioscience; 1/100 dilution-48 min), rabbit anti-Bcl 2 alpha polyclonal antibody (E17980, Spring Bioscience; 1/200 dilution-44 min), rabbit anti-Bax monoclonal antibody (Clone SP47, M3470, Spring Bioscience; 1/50 dilution-32 min), rabbit antihuman bcl-xL polyclonal antibody (E3370, Spring Bioscience; 1/200 dilution-16 min), and rabbit antihuman cyclin D1 monoclonal antibody (Clone SP4, M3040, Spring Bioscience; 1/100 dilution-32 min) were used. Amplification was performed only with p21 and Bcl-2. Positive and negative controls were used in each immunohistochemical staining.

The preparations with immunohistochemical staining were screened by 3DHISTECH Kft. in Budapest, Hungary and digital images that would enable computed analysis were created.

### 2.4. Evaluation of immunohistochemical outcomes

All evaluations were performed via computer using the Panoramic Viewer 1.14 and DensitoQuant 1.14 applications of the 3DHISTECH TMA Module 1.14 program (Budapest, Hungary). Initially, orientation of images was provided using the 3DHISTECH Panoramic Viewer 1.14 application. Thereafter, Bcl-2, Bcl-xL, and Bax cytoplasmic staining, as well as p53 nuclear and cytoplasmic staining, were analyzed using a DensitoQuant 1.14 application. Three lesion areas with similar size and with the densest staining were marked on each image. The highest staining percentage in these 3 areas was considered in this study.

Evaluation of p16, p21, p27, and cyclin D1, which cause nuclear staining, was performed on digital images with 100 $\times$  and 400 $\times$  magnifications by 2 separate pathologists. Three consequent areas including 200 cells were counted and the highest staining percentage was taken as the basis for study.

### 2.5. Statistical analysis

Data were analyzed using the SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Correlation was analyzed using Pearson and Spearman correlation analyses. The Kruskal–Wallis test was used for comparison of more than 2 independent groups. The Mann–Whitney U test was used to compare differences between 2 independent groups when the dependent variable was either ordinal or continuous. The Wilcoxon test was used when comparing 2 related samples, matched samples that came from the same population.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Age and sex

The age of patients ranged between 17 and 70 years (mean:  $44.13 \pm 12.89$  years) in the FA group (the majority (36.7%) in the fifth decade), 23 and 70 years (mean:  $47.43 \pm 14.97$  years) in the FC group (majority in the third (18.8%) and sixth (18.8%) decades), and 21 and 59 years (mean:  $44.00 \pm 11.25$  years) in the AN group (majority (40%) in the sixth decade). Twelve (75%) of the FC, 22 (73.3%) of the FA, and 15 (75%) of the AN patients were females and 4 (25%) of the FC, 8 (26.7%) of the FA, and 5 (25%) of the AN patients were male.

With regard to the age distribution of all groups, it was determined that the majority of the patients were adults and females. No significant difference was found between the groups in terms of age ( $P > 0.05$ ).

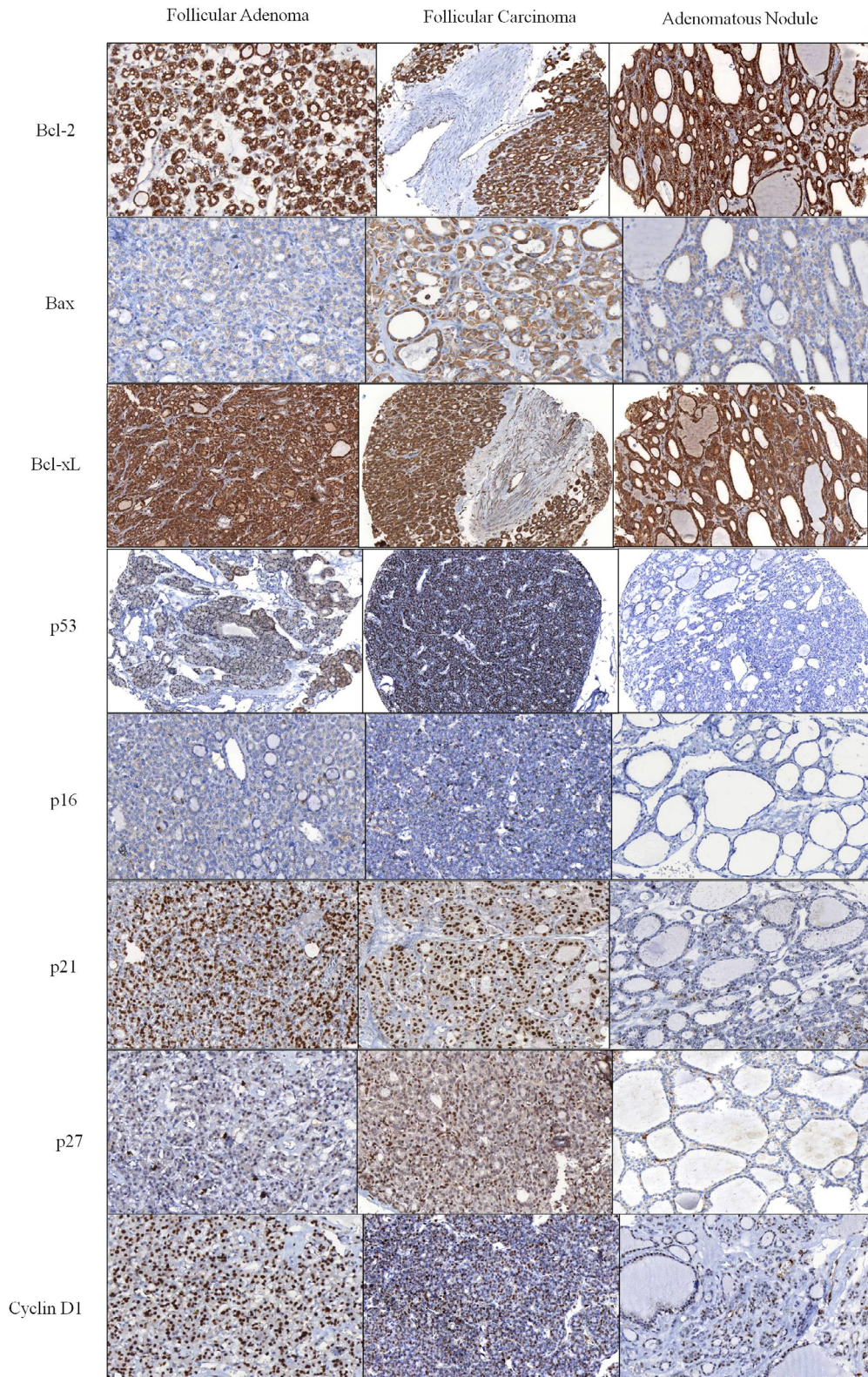
### 3.2. Results of immunohistochemical staining

It was detected that Bcl-2, Bax, and Bcl-xL caused cytoplasmic staining, p53 caused cytoplasmic and nuclear staining, and p16, p21, p27 and cyclin D1 caused nuclear staining in the neoplastic and nonneoplastic thyrocytes (Figure). The mean staining percentage of 8 immunohistochemical markers among groups is demonstrated in the Table.

The percentage of Bcl-2 and Bcl-xL staining was high in all groups. The highest Bax staining percentage was in the FC group, although more than half of overall lesions were negative with Bax. p53 was the lowest in the AN group and the highest in the FC group. p16 was significantly higher in the FC group than the other groups. p21 staining was lower in the AN group than in the FA and FC groups. The number of cases with positive p27 was lower in the AN group than the neoplastic groups but p27 staining percentages of the groups were close to each other. The highest cyclin D1 staining percentage was in the FA group, whereas the lowest percentage was in the AN group.

The percentages of p53, p16, and p21 staining showed significant differences between the AN group and the other 2 groups containing neoplastic lesions ( $P < 0.05$  for each). There were significant differences among the 3 study groups with these stainings ( $P < 0.05$  for each).





**Figure.** Cytoplasmic staining of Bcl-2, Bax, and Bcl-xL; nuclear and cytoplasmic staining of p53; nuclear staining of p16, p21, p27, and Cyclin D1.

**Table.** The mean staining percentage of immunohistochemical markers among groups.

Immunohistochemical marker	Follicular adenoma	Follicular carcinoma	Adenomatous nodule
Bcl-2	71.69 ± 24.58	88.56 ± 9.84	85.72 ± 12.50
Bax	9.51 ± 17.72	33.38 ± 36.47	3.88 ± 7.26
Bcl-xL	89.95 ± 17.90	96.02 ± 6.92	94.24 ± 7.88
p53	7.14 ± 16.14	28.89 ± 35.41	1.38 ± 2.00
p16	10.96 ± 20.71	32.33 ± 38.49	1.05 ± 1.35
p21	25.39 ± 20.53	34.68 ± 22.02	12.89 ± 13.87
p27	3.62 ± 4.49	3.26 ± 5.29	3.60 ± 9.30
Cyclin D1	32.26 ± 26.57	20.00 ± 14.71	19.15 ± 18.09

There were significant differences between benign (FA and AN) and malignant (FC) groups in percentages of bcl-2, Bax, and p16 staining ( $P < 0.05$  for each).

No significant difference was observed among the groups in terms of bcl-xL, p27, and cyclin D1 staining ( $P > 0.05$ ).

In the FC group, there was a highly positive correlation between the percentages of Bcl-2 and Bcl-xL staining ( $r = 0.50$ ). In the FC and FA groups, a highly positive correlation was found between the percentages of Bax and p53 staining ( $r = 0.79$  and  $r = 0.85$ , respectively).

#### 4. Discussion

Age and sex distribution of the study groups was in line with the classical information on thyroid lesions (1,2); the majority of the patients were adult females. No significant difference was determined among the groups in terms of age ( $P > 0.05$ ).

In line with the literature, both benign and malignant samples in all study groups showed cytoplasmic Bcl-2 staining (3–7) with the highest staining percentage in the FC group. Bcl-2 stainings being significantly different in the FA and AN groups (represented as benign lesions) as compared to the FC groups (represented as malignant lesions) can be interpreted as demonstrating the way that the apoptosis pathway, in which Bcl-2 plays a role, is different between benign and malignant lesions. The fact that the FC group showed a higher Bcl-2 expression than the other groups indicates prolonged lifetime in tumor cells and the role of Bcl-2 in carcinogenesis.

Approximately one-third of the FC group and more than half of the lesions in the other groups showed no Bax staining. The low percentage of immunochemical Bax staining in the AN group is consistent with the literature (5,8,9).

In the FC and FA groups, a highly positive correlation was found between Bax and p53 ( $r = 0.79$  and  $r = 0.85$ ,

respectively). Similar correlations have been described in the literature and it has been reported that Bax also shows a high staining percentage in thyroid cancers, in which the percentage of p53 staining is high (4,6,8). Correlation between Bax and p53 suggests that the Bax gene and p53, which is the transcriptional activator of Bax, together have an effective role in the pathogenesis of neoplastic lesions.

It has been suggested that Bcl-xL shows no or weak staining in regular follicular cells and is more highly expressed in thyroid carcinomas originating from follicular cells (7,10). In the present study, the facts that there was no significant difference among the groups (despite the highest staining percentage being in the FC group) and that all of the FA, FC, and AN groups showed positive Bcl-xL staining and all had a high staining percentage were inconsistent with the literature.

A highly positive correlation ( $r = 0.50$ ) between the percentages of Bcl-2 and Bcl-xL staining suggested a shared antiapoptotic effect.

With regard to the overall members of the Bcl-2 gene family, it was concluded that Bcl-2, Bax, and Bcl-xL showed extensive positivity in FC, findings that were largely consistent with the literature, and all members of this gene family, Bcl-2 being most prominent, had a role in the pathogenesis of tumors derived from thyroid follicular epithelial cells.

It has been suggested in the literature that normal thyroid tissue and benign thyroid lesions show no p53 staining and that p53 mutation is seen in 15% of overall thyroid carcinomas, being higher in undifferentiated and poorly differentiated thyroid carcinomas. Reported p53 positivity varies between 30% and 90% in FCs and between 15% and 33.3% in FAs (8,11–17).

The results of the present study concerning p53 are consistent with the literature (3,5,8,11,13–23) with remarkably high p53 staining in the carcinoma group. The number of stained lesions and the staining percentage



were significantly lower in the AN group compared to the neoplastic groups. Consideration of these findings, together with those reported in the literature, suggests that p53 is a predictor for malignancy and has a role in tumor pathogenesis.

While previous research found no p16 staining in nonneoplastic lesions or normal thyroid tissue, p16 positivity was observed in thyroid papillary carcinoma and FCs. It has been suggested that p16 plays an important role in transforming the benign form into the malignant form in follicular thyroid cells (24–26). Significantly low p16 staining in the AN group compared to the FA group and particularly the FC group and, in contrast, the high p16 staining in the FC group are in line with information in the literature and suggest that p16 has a role in the tumorigenesis of neoplasms derived from follicular epithelial cells, particularly in malignant transformation.

p21 staining, observed in all the FA and FC lesions, is rarely observed in normal thyroid cells and hyperplasia but is observed at 10%–28% in FAs. Although there are studies reporting that p21 has no importance in patient survival, there are also studies suggesting that it has importance in tumor aggression and is high in patients with fatal progression (27–30). Staining characteristics with p21 in the present study are consistent with those reported in the literature and the difference between neoplastic and nonneoplastic lesions in particular attracts attention to the role of p21 in the pathogenesis of follicular neoplasm.

Nuclear p27 positivity has been reported to be higher in normal thyroid tissue and multinodular goiter than in tumoral tissues. Stronger positivity has been detected in cases of papillary hyperplasia than in cases of thyroid papillary carcinoma and it has been suggested that it could be helpful in differentiating benign from malignant papillary proliferations. Higher p27 positivity has been observed in

FA cases as compared to the FC and TPC-follicular variants (31–34). It has been reported that loss of p27 expression indicates tumor aggression, poor differentiation, and metastasis capacity in thyroid carcinomas (32–35). In the present study, the results concerning p27 did not completely reflect the loss of p27 expected in thyroid carcinomas. Higher p27 staining was also reported in oncocytic lesions as compared to nononcocytic tumors (36–38). In the present study, the reason for higher p27 staining than expected instead of loss of p27 might be the higher staining percentage in the oncocytic lesions in both the FA and FC groups. Moreover, the absence of poorly differentiated cases in the FC group might have been effective in obscuring the loss of p27 expression.

Cyclin D1, which has a nuclear staining character, is not observed in normal thyroid cells. Results concerning staining in hyperplastic lesions are contradictory. Positivity has been demonstrated in FA, FC, thyroid papillary carcinoma, and Hurtle cell carcinoma (39–46). In the present study, there was no case without cyclin D1 staining in the FA, FC, and AN groups and no significant difference was found among the groups in terms of cyclin D1 staining. Although cyclin D1 staining in the present study was observed in all cases, expression in malignant lesions is not as high as suggested.

In conclusion, the present study revealed that cell cycle modulators, led by the Bcl-2 family, played an important role in the pathogenesis of follicular cell neoplasms, particularly with significant roles of p53, p16, and p21 in the pathogenesis of FCs.

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