

## Association of Pro-apoptotic *Bad* Gene Expression Changes with Benign Thyroid Nodules

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**Abstract.** *Background/Aim: This study aimed to investigate the role of the mitochondrial apoptotic pathway in benign thyroid nodules. Materials and Methods: Paired samples of nodular and normal tissues were collected from 26 patients with nodular goiters undergoing thyroidectomy. Variable expression of Bcl-2, Bax and Bad genes were evaluated by quantitative PCR. Results: Expression level of Bad gene in nodules was found to be significantly decreased compared to normal tissues ( $p=0.049$ ). A positive correlation was observed between nodule size and Bad expression levels (correlation coefficient=0.563,  $p=0.004$ ); and this correlation was stronger in hot nodules ( $n=18$ , correlation coefficient=0.689,  $p=0.003$ ). No significant difference was observed between nodular and normal tissue expressions of Bax and Bcl-2. Conclusion: These results suggest that Bad expression correlates with the size of benign thyroid nodules and also its relatively lower expression in nodules, warrant further investigation.*

The etiology of benign thyroid nodules is generally believed to be multifactorial in origin. Environmental factors like iodine deficiency, genetic predisposition and endogenous factors including gender may contribute to nodule development (1). A monoclonal origin detected in the

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majority of thyroid nodules implicates a benign nodular development from a single mutated thyroid cell. Activating thyrotropin receptor and alpha subunit of the G protein ( $G_s\alpha$ ) mutations are prevalent in autonomously functioning thyroid nodules (AFTNs), but knowledge concerning the molecular etiology of benign cold thyroid nodules is limited (2-5).

In normal tissues, total cell mass is maintained by the dynamic balance between cell proliferation and programmed cell death, apoptosis. Several control systems maintain homeostasis by both stimulating and inhibiting apoptosis (6). Death receptors (DRs), which are proteins related to the TNF receptors (TNFRs), also contribute to the maintenance of homeostasis by modulating the apoptotic threshold of the cell in response to a variety of chemical substances (7).

The apoptotic machinery of the cell apart from the DRs includes adaptor molecules, caspases and the proteins of the Bcl-2 family. Bcl-2 family includes both pro-survival and pro-apoptotic members (7). Its pro-survival members (Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1) oppose two proapoptotic groups, known as the Bax group and the BH3-only proteins. Members of the Bax group (Bax, Bak and Bok) are structurally similar to Bcl-2 and bear three 'BH' (Bcl-2 homology) domains, whereas the BH3-only proteins, which include Bim, Bad, Bid, Bik, Bmf, Puma, Noxa and Hrk, share only the BH3 interaction domain (8).

Apoptosis provides protection from the possible consequences of increased or uncontrolled cell proliferation, which could lead to hyperplasia or neoplasia (9). Fas and TNF-related apoptosis inducing ligand (TRAIL) dependent apoptotic pathways are the two most frequently investigated pro-apoptotic pathways with relevance to thyroid homeostasis (10-12). Recent evidence has emphasized on the importance of apoptosis in the pathogenesis of thyroid cancer and autoimmune thyroid disorders (13), and cell cycle modulators controlled by the Bcl-2 family proteins were shown to be important in the pathogenesis of thyroid follicular neoplasms (14). However, the role of apoptosis in

the molecular pathogenesis of benign thyroid nodules has not been extensively investigated. More subtle impairments of growth inhibition compared to those observed in malignant conditions may participate in the imbalanced tissue homeostasis that results in nodular goiter; and thyroid cells with advantageous mutations can further proliferate and develop into benign thyroid nodules.

The purpose of this pilot study was to investigate the role of Bcl-2 apoptotic pathway in the pathogenesis of benign thyroid nodules by measuring mRNA levels of pro-survival Bcl-2 and pro-apoptotic Bax and Bad genes in paired samples of nodular and normal (non-nodular) appearing thyroid tissues.

## Materials and Methods

**Patients and tissues.** The local ethics committee approved the protocol of this study, and a written informed consent was obtained from all participants. Thyroid tissue samples were collected from 52 patients with solitary or multinodular goiters undergoing surgery for the removal of thyroid nodules. All patients were negative for anti-thyroglobulin and anti-thyroperoxidase antibodies. Specimens collected from dominant nodules and apparently normal non-nodular thyroid tissues from each patient were snap-frozen in liquid nitrogen before RNA isolation. During histopathologic examination, 26 of 52 patients were excluded from the study either due to thyroid cancer or due to lymphocytic infiltration. Paired samples of the nodular and normal tissues of the remaining 26 patients comprised the study group. The nodules were classified as cold or hot based on their scintigraphic features. Nodule sizes were measured by both preoperative ultrasound and postoperative macroscopic examination, and the results of the latter were recorded.

**Quantitative RT-PCR.** Total RNA was extracted from the samples using the RNeasy Fibrous Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed in a 20- $\mu$ l reaction volume containing 5  $\mu$ g/ml random hexamers, 0,5 mM dNTP, 0,01 M DTT, 1 U/  $\mu$ l ribonuclease inhibitor, 2.5 U/ $\mu$ l reverse transcriptase (all enzymes and chemicals were supplied from MBI Fermentas, Germany). The PCR conditions for the cDNA were as follows: 10 min at 70°C, 1 h at 37°C and 5 min at 99°C and was stored at -20°C prior to quantitative PCR analysis.

Expression levels of the Bcl-2, Bax and Bad genes were analyzed by quantitative PCR using LightCycler (Roche Diagnostics GmbH, Germany). We have used the SYBR Green 1 dye (Roche, Mannheim, Germany) binding method. Oligonucleotide primers for the genes analyzed and for  $\beta$ 2-microglobulin (B2M) as endogenous control were as follows: Bcl-2 – forward 5'-AGG AAG TGA ACA TTT CGG TGA C-3' and Bcl-2 – reverse 5'-GCT CAG TTC CAG GAC CAG GC-3', Bax – forward 5'-GAT GAT CCA TGT TTT CAG CGA C-3' and Bax – reverse 5'-CTC CAC AAA CCC ATC CCA G-3', Bad – forward 5'- GAG TGA GCA GGA AGA CTC CAG C-3' and Bad – reverse 5'-TCC ACA AAC TCG TCA CTC ATC C-3', B2M – forward 5'-TGC CGT GTG AAC CAT GTG AC-3' and B2M – reverse: 5'-ACC TCC ATG ATG CTG CTT ACA-3'.

The following amplification program was used: One min at 95°C, followed by 40 cycles consisting of 10 sec at 95°C, 5 sec at 58°C, and 20 sec at 72°C. Melting curve analysis consisted of 0 sec at

95°C, 10 sec at 65°C, and 10 sec at 95°C. Standard curves were generated for each amplification using serial dilutions of a cDNA mixture expressing B2M. Expression of each gene was calculated with a program found in LightCycler software. Relative expression units (REUs) were calculated using expression of the B2M gene as endogenous control.

**Statistical analysis.** Results are expressed as the mean $\pm$ SD, median (minimum-maximum). Expression levels were compared using nonparametric Wilcoxon signed-rank test. Correlation of Bcl-2, Bax and Bad gene expression with clinical parameters was analyzed using Spearman test and  $p < 0.05$  was considered statistically significant.

## Results

We analyzed normal and nodular thyroid tissues of 26 patients to investigate the role of Bcl-2 mitochondrial apoptotic pathway in the pathogenesis of benign thyroid nodules, and 18 of the nodules were classified as hot and 8 as cold. Table I summarizes the characteristics of each patient included in the study.

Relative mRNA expression levels of the studied Bcl-2, Bax and Bad genes are given in Table II. Analysis of the entire set of benign nodules (n=26) revealed that Bad mRNA expression levels were significantly lower than those in apparently normal thyroid tissues from the same patients ( $p=0.049$ ). There was no significant difference observed in the levels of Bax and Bcl-2 mRNA expressions between the paired nodular and normal tissues.

In the subgroup analysis of patients with hot (n=18) or cold (n=8) nodules, no significant differences were observed in the expression levels of Bcl-2, Bax and Bad genes between the normal and nodular thyroid tissues.

A strong positive correlation was observed between nodule size and Bad mRNA expression levels of the entire set of nodules (correlation coefficient=0.563,  $p=0.004$ ). This correlation was stronger when only hot nodules were considered (correlation coefficient 0.689,  $p=0.003$ ).

## Discussion

In this pilot study, we observed decreased mRNA expression of pro-apoptotic Bad gene in the nodules compared to the paired normal thyroid tissues from the same patients despite the positive correlation between the size of the nodule and the amount of Bad mRNA.

A sensitive quantitative RT-PCR method was selected to detect subtle changes, which can be associated with a benign nodule development. We expected that these expression changes would not be prominent enough to be detected like those changes observed when malignant thyroid nodules were compared with benign nodules (14). In agreement with a previous study using immunohistochemical methods, no significant difference was observed in the expression levels of Bcl-2 and Bax genes between the normal and benign

Table I. Clinical and histological characteristics of the study group.

No	Age (years)	Gender	TSH value before surgery (mIU/l)	Nodule diameter (mm)	Scintigraphy	Diagnosis	Treatment before surgery	Histopathology
1	73	F	0.571	51	Cold	MNG	None	AN
2	74	F	0.697	45	Cold	MNG	None	AN
3	70	F	2.66	34	Cold	MNG	None	FA
4	41	F	1.82	23	Cold	MNG	None	AN
5	54	M	1.15	38	Cold	SN	None	FA
6	59	F	2.20	12	Cold	MNG	None	AN
7	38	M	0.277	40	Cold	MNG	None	AN
8	19	F	0.686	20	Cold	SN	None	AN
9	53	F	0.005	45	Hot	TMNG	PTU	AN
10	48	M	0.007	23	Hot	TMNG	MMI	AN
11	36	M	0.462	47	Hot	SN	None	AN
12	54	F	0.071	38	Hot	TMNG	PTU	AN
13	53	M	0.023	49	Hot	TA	None	AN
14	52	F	0.149	28	Hot	TMNG	None	AN
15	53	F	0.018	28	Hot	TA	None	AN
16	57	M	0.006	45	Hot	TMNG	MMI	AN
17	60	M	0.022	33	Hot	TMNG	MMI	AN
18	50	M	0.248	43	Hot	TMNG	MMI	AN
19	40	M	0.499	30	Hot	MNG	None	AN
20	57	F	0.054	25	Hot	TMNG	None	AN
21	72	F	0.008	27	Hot	TMNG	PTU	AN
22	56	F	0.013	45	Hot	TMNG	MMI	AN
23	39	M	0.44	14	Hot	MNG	None	AN
24	51	M	0.019	35	Hot	TMNG	MMI	AN
25	57	F	0.186	25	Hot	TMNG	MMI	AN
26	52	F	0.153	32	Hot	TMNG	None	AN

M: Male; F: female; MNG: multinodular goiter; SN: solitary nodule; TMNG: toxic multinodular goiter; TA: toxic adenoma; PTU: propylthiouracil; MMI: methimazole; AN: adenomatous goiter; FA: follicular adenoma.

nodular thyroid tissues (15). However, the observation of a positive correlation between the level of Bad gene expression and nodule size as well as a relative decrease in its expression may suggest that Bad may have a regulatory role in the development of benign thyroid nodules and an imbalance in its expression may contribute to the pathogenesis of nodule development.

Bad (Bcl-2 antagonist of cell death, Bcl-2 binding component 6) is a small pro-apoptotic protein consisting of 168 amino acids (16). It plays an important role in connecting growth signaling pathway to cell death pathway. Bad deficient mice are developmentally normal by most criteria, however, isolated thymocytes, fibroblasts, and epithelial cells from Bad deficient mice were found to be resistant to cell death induced by growth factor withdrawal, consistent with a pro-death function of Bad under these conditions. Furthermore, Bad deficient mice develop B cell lymphomas and other malignancies, and this propensity is dramatically increased by exposure to limited  $\gamma$ -irradiation. The apoptotic activity of Bad is inhibited by phosphorylation (17).

Table II. Expression of Bcl-2 family apoptotic genes in thyroid tissues (n=26).

Gene	Tissue	Relative Expression Unit Mean $\pm$ SD (median (minimum-maximum))
<i>Bcl-2</i>	Normal	0.333 $\pm$ 0.437 (0.116 (0.006-1.541))
	Nodule	0.345 $\pm$ 0.643 (0.115 (0.021-3.002))
<i>Bax</i>	Normal	0.042 $\pm$ 0.029 (0.035 (0.005-0.117))
	Nodule	0.066 $\pm$ 0.077 (0.035 (0.0002-0.328))
<i>Bad</i>	Normal	0.048 $\pm$ 0.097 (0.020 (0.003-0.485))
	Nodule	0.017 $\pm$ 0.012 (0.014 (0.003-0.057))

Mezosi and colleagues examined TRAIL and FasL- induced apoptotic pathways in primary thyroid cells from 17 patients with nodular goiter, using 10 normal thyroids as controls (18).

Although normal thyroid cells could be sensitized *in vitro* to TRAIL and FasL by inflammatory cytokines, the majority of goiter samples showed resistance to death ligands after cytokine pretreatment. The resistance of goiter cells to TRAIL was not caused by the altered surface expression of death receptors or the overexpression of well-known intracellular inhibitors such as cFLIP, Bcl-2, or inhibitor of apoptosis proteins raising the possibility that other inhibitors or a down-regulation of pro-apoptotic proteins might be responsible. In a subset of goiters they found increased proteasome activity, and they suggested that this increase might be responsible for the down regulation of pro-apoptotic proteins as most intracellular proteins are degraded by the proteasome complex (18). At another study of AFTNs using microarray technology, Wattel and colleagues showed overexpression of anti-apoptotic genes and under-expression of pro-apoptotic genes compatible with their low apoptosis rate (19).

Recent studies suggest a role for the phosphoinositide 3-kinase (PI3K)/Akt and Bad gene pathway in the development of papillary thyroid cancer (20, 21), which can be a supportive finding for the critical role of Bad in the survival of thyroid cells and nodulogenesis. The correlation of *Bad* gene mRNA expression with nodule size further supports its importance as a regulatory protein in thyroid cell apoptosis, however its expression may not be sufficient enough to inhibit the development of nodules in some patients with genetic or epigenetic alterations leading to an imbalance with relatively lower Bad gene expression.

This pilot study has several limitations, mainly due to lack of confirmatory findings using additional methods to document the expression changes. As expected, the observed changes in the expression of the Bad gene were not so strong enough to be documented by immunohistochemical methods in benign nodules, and the findings of this study are promising enough to be studied in larger groups with additional sensitive tools to detect minimal changes in gene expression.

## Conclusion

The positive correlation of Bad expression with nodule size and a relative decrease in the mRNA expression level of Bad in benign thyroid nodules suggest that Bad may be an important regulator of thyroid cell apoptosis. These preliminary findings may indicate a role for deregulated Bad gene expression in the pathogenesis of thyroid nodules and warrant further investigations in larger series of patients and experimental models.

## Conflicts of Interest

The Authors declare that they have no conflicts of interest relevant to this study. No financial support other than Istanbul University Research Fund (T-285/18062003) was received.

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