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Characterization of Microarray Gene Expression Profiles of Early Stage Thyroid Tumours

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Abstract. *Background: Microarray analysis offers the opportunity of screening transcriptional expression profile of neoplastic cells on a genomic level. Defining consistent changes in gene expression pattern of tumours enables the detection of genes essential for tumorigenesis and might provide biomarkers to early recognition of malignant behaviour and new therapeutical targets. Patients and Methods: A high-density oligonucleotide array with 20,000 human gene-specific oligonucleotide was used to analyze benign and early-stage malignant thyroid tumours of epithelial origin: follicular adenoma, follicular carcinoma and papillary carcinoma, compared to normal thyroid tissue. Results: Significant expression differences of 279 genes – underexpression of 252 and overexpression of 27 genes – were found. The overlapping genes of the different histological types were examined extensively. Among these genes a limited set acting on the same transcriptional pathway, through NF-κB, were found. Conclusion: The role of overlapping genes in histologically different tumours has not been clarified, but might represent early or pivotal steps of carcinogenesis. All investigated histiotypes of tumours contained significantly modulated genes acting on the NF-κB regulatory pathway. Our findings suggest that modulation of NF-κB signalling plays a crucial role in early thyroid carcinogenesis.*

The most frequent endocrine neoplasms are the tumours of the thyroid gland, with an incidence of 9 per 100,000 cases per year in industrialized countries (1). Benign thyroid nodules are far more common. An estimated 5-10% of the population develops a clinically significant thyroid nodule during their lifetime (2). Therefore distinguishing benign from malignant nodules is a frequent diagnostic dilemma in the clinical practice. Importance of early recognition of thyroid malignancies lies in the fact that in the nodular form of the disease surgical treatment could provide complete recovery if the diagnosis is established before the development of local invasion and metastasis. The evaluation of thyroid nodules by fine-needle aspiration (FNA) has been a standard for almost 30 years. However, the quality and quantity of the cytological samples taken by FNA are frequently not suitable for cytological evaluation. Invasiveness, metastatic features and many other molecular features of tumours are not discernible on cytology and only after surgical resection and histological evaluation can they be recognised (3).

High throughput technologies, such as microarray, offer the opportunity of screening the entire transcriptional expression profile of neoplastic cells on a genomic level. Defining consistent changes in the gene expression pattern of tumours might lead to detection of genes essential for tumour development and might provide new biomarkers to classify tumours based on molecular features, early molecular identification of malignant behaviour, as well as new targets for therapy (4).

Patients and Methods

Tissue samples. Tumour tissue samples were obtained from patients with benign and clinically early-stage malignant thyroid tumours (T1, N0, M0) undergoing surgery at the First Department of Surgery in Debrecen University. All tumour samples were obtained with the permission of institutional review boards and informed consent of the patients. Dissected samples were frozen immediately after surgery at stored at –80°C. Histopathological classification was confirmed by an endocrine pathologist. Tumour samples of follicular adenoma (n=8), follicular carcinoma (n=7), papillary carcinoma (n=10) and normal thyroid tissue (from patients who underwent total thyroidectomy n=20) were selected for our investigations.

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RNA isolation. Total RNA was isolated from 40 mg of each tissue sample with NucleoSpin RNA purification kit (Macherey-Nagel, Dürren, Germany) according to the manufacturer's instructions. The quality and quantity of all RNA preparations were assessed by agarose gel electrophoresis and spectrophotometry (NanoDrop, Rockland, DE, USA). Total RNA was used both for microarray analysis and quantitative real-time PCR.

Microarray design. A total of 20,000 human gene-specific, amino-modified oligonucleotides were purchased (Sigma-Operon, Budapest, Hungary) and arrayed onto PXM oligonucleotide slides (Full Moon Biosystems, Sunnyvale, CA, USA) using a MicroGrid Total Array System (BioRobotics, Cambridge, UK) spotter. DNA elements were deposited in duplicate in distinct areas of the array. After printing, slides were incubated in a humid chamber for 14 hours at 42˚C.

Microarray preparation and hybridization. Two micrograms of total RNA were reverse transcribed using a poly-dT primed Genisphere Expression Array 900 Detection system (Genisphere, Hatfield, PA, USA) in 20 μl total volume using 20 U RNAsin (Fermentas, Vilnius, Lithuania), 1x first strand buffer and U of RNAse H(–) point mutant M-MLV reverse transcriptase (Fermentas). All the other probe preparation steps were carried out according to the manufacturer's instructions (Genisphere). Both the first step cDNA hybridization and the second step capture reagent (2.5 μl of each Cy5 and Cy3) hybridization were carried out in a Ventana hybridization station (Ventana Discovery, Tucson, AZ, USA) using the antibody protocol. First hybridization was performed at 40˚C for 6 hours in FGL2 hybridization buffer (10x Denhart solution, 0.25M sodium phosphate buffer pH 7.0, 1 mM ethylene-diamine-tetraacetic acid, 1x salt sodium citrate, 0.5% sodium dodecyl sulphate) then 2.5 μl of each Cy5 and Cy3 capture reagents were added to the slides in 200 μl "Chiphyb" hybridization buffer (Ventana) and they were incubated at 42˚C for 2 hours. After hybridization, the slides were washed in 0.2x salt sodium citrate twice at room temperature for 10 minutes, then dried and scanned.

Scanning and data analysis. Each array was scanned under a green (543 nm for Cy3 labeling) or a red laser (633 nm for Cy5 labeling) using a ScanArray Lite (GSI Lumonics, Billerica, MA, USA) scanning confocal fluorescence scanner. Scanned output files were analyzed using the GenePix Pro 5.0 software (Axon Instruments Inc., Foster City, CA, USA). For each channel, the median values of feature and local background pixel intensities were determined. The background corrected expression data was filtered for flagged spots and weak signal. Technical replicates on the same array were averaged. Data was excluded in case where technical replicates were significantly different. Normalization was performed using the print-tip LOWESS method. The one-sample *t*-test was used in order to determine the genes to be regarded as regulated. Expression ratios were log-transformed to fulfil the *t*-test's requirement for a normal distribution. Genes for which the mean of log-ratios across the biological replicates was equal to zero at a significance level $\alpha=0.05$ were considered to have an unchanged expression. On the other hand, genes having a *p*-value smaller than α and an average fold-change (increase or decrease) of the four data points of at least 2.0-fold were considered as modulated genes.

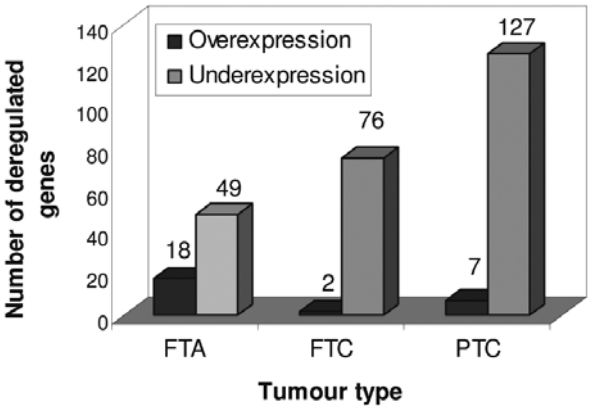

Figure 1. *Number of significantly deregulated genes found in the different hystiotypes of thyroid tumors. FTA: follicular thyroid adenoma, FTC: follicular thyroid carcinoma, PTC: papillary thyroid carcinoma.*

From among the group of significantly modulated genes, attention was focused on those showing higher than two-fold expression change constitutively in all of the tumour samples in a histopathological group.

Gene expression analysis. In the case of 5 genes, gene expression changes observed on microarray were confirmed by quantitative real-time (QRT) PCR. Total RNA from each sample (2 μg) was reverse transcribed in the presence of poly (dT) sequences and was used as template in the QRT-PCR. Primers were designed using the PrimerExpress software (Applied Biosystem, Foster City, CA, USA). Reactions were performed in a total volume of 20 μl containing 0.2 mM of dNTP, 1xPCR reaction buffer (ABGene, Epsom, UK), 5 pmol of each primer, 4 mM of $MgCl_2$, 1x SybrGreen I (Molecular Probes, Eugene, Oregon, USA) and 0.5 units of thermostat Taq DNA polymerase (ABGene). Quantitative PCR was performed using a RotorGene 3000 instrument (Corbett Research, Sidney, Australia) according to the SybrGreen protocol. Curves were analyzed by RotoGene software (Corbett Research) using dynamic tube and slope correction methods, ignoring data from cycles close to baseline. Relative expression ratios were normalized to the geometric mean of two housekeeping genes, GAPDH and hypoxanthin phosphorybosyltransferase. Expression ratios were calculated using the Pfaffl method (5). All the PCRs were performed four times in separate runs. Regulation was confirmed in the correct direction for each of the 5 genes.

Results

Significant expression differences of 279 genes, underexpression of 252 and overexpression of 27 genes, were found in the thyroid tumour samples of follicular adenoma, follicular carcinoma and papillary carcinoma compared to the normal thyroid tissue. Underexpressions were more expressed in all tumour samples than overexpressions and showed an increase in number in the malignant histiotypes (Figure 1). While the overexpressions

Table I. *Overexpressions in thyroid tumors.*

ID	Name	Log. ratio	*p*-value
	Follicular thyroid adenoma		
AB044385_1	Transmembrane molecule with thrombospondin module	2.225	0.001
AF152323_1	Protocadherin gamma A3	2.417	<0.05
AF387908_1	MHC class I antigen	2.087	0.010
BC000814_1	TG-interacting factor (tale family homeobox)	2.263	<0.05
BC003382_1	Sorting nexin 2	2.166	0.006
BC011027_1	Ectodysplasin A isoform 1 EDA1	2.285	0.001
NM031942_1	Cell division cycle associated protein 7 isoform 1	2.167	0.009
NM080798_1	Alpha 1 type XIII collagen isoform 2	2.460	<0.05
XM012425_1	Lysyl oxidase-like 1	2.196	<0.05
XM030577_1	Potential phospholipid-transporting ATPase IIa	2.203	0.001
XM051590_1	Nuclear pore protein GP210 precursor	2.004	<0.05
	Follicular thyroid adenoma/ follicular thyroid carcinoma		
XM166349_1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells, NF-κB	3.426/2.066	0.021/0.007
	Follicular thyroid carcinoma		
XM170650_1	Riken cDNA 4921522e24	2.237	0.016
	Papillary thyroid carcinoma		
AB083586_1	Putative G-protein coupled receptor	2.576	<0.05
AJ250014_1	Cyclin D1, CYLD1	2.235	<0.05
BC015753_1	GRO2 oncogene	2.088	<0.05
D89501_1	Salivary proline-rich protein-B1, PB1	2.279	0.023
K03208_1	Proline-rich protein BstNI subfamily, PRB2	2.166	0.001

were specific for each tumour histiotype (Table I), the underexpressed genes exhibited many overlaps between the tumour groups (Tables II and III). Moreover 25 out of 49 underexpressed genes found in follicular adenoma exhibited overlaps with the malignant histological types.

Analysing these overlapping genes between the benign and malignant histiotypes, the underexpression of *Bruton's tyrosine kinase (BTK), G protein coupled receptor 30 (GPR 30), apelin, E-cadherin (CDH9)* and *CDP-diacylglycerol (CDP-DAG)* were identified in follicular adenoma and follicular carcinoma, while the underexpressed genes in follicular adenoma and papillary carcinoma included *interleukin 12 receptor β-2 (IL12Rβ-2)* (Table II).

Overlaps of underexpressions in the malignant histiotypes (follicular carcinoma and papillary carcinoma) involved *phospholipase D1 (PLD1)* and *mitogen-activated protein kinase 10 (MAPK10)*. We also found some novel genes down-regulated in follicular and papillary thyroid carcinoma, which had not been reported yet in thyroid malignancies, such as *SOX21* and *NIL-2-A*. However the consequent significant underexpression of three particular genes in all investigated tumour groups was remarkable: *Peroxisome proliferator-activated receptor-gamma (PPARγ)*, *eosinophil-derived neurotoxin and urotensin II* (Table III).

We found small number of constitutively up-regulated genes in the investigated tumour histiotypes and this number showed further decrease in the malignant groups, whereas these up-regulated genes were characteristic to the different tumour histiotypes. The most considerable was the *EDA1* overexpression identified in follicular thyroid adenoma and the *cyclin D1* (*CYLD1*) overexpression represented in papillary carcinoma. Among the overexpressed genes just one was found to be significantly regulated in all follicular tumour types: *NF-κB*.

Discussion

Microarray analysis enables us to take a genome-wide cross-sectional snapshot of tumours. Analysis and comparison of these molecular snapshots makes it possible to identify characteristic steps of deregulation and dedifferentiation in tumorigenesis. Both the advances and the limitations of this genome-wide screening arise from the nature of the methodology. It can reflect a momentary picture of a global network of all transcriptional events, but is unable to represent the dynamically changing molecular process of tumour development. For that reason in this present investigation we compared the microarray pattern of early-stage thyroid tumours looking not particularly for

Table II. *Genes overlapping in underexpression FTA: follicular thyroid adenoma, FTC: follicular thyroid carcinoma, PTC: papillary thyroid carcinoma.*

ID	Name	Log. ratio	*p*-value	Log. ratio	*p*-value
		FTA		FTC	
AF153762_1	Bruton's tyrosine kinase, BTK	–2.025	0.0075	–2.661	0.0140
AF208694_1	Impact	–2.594	0.0013	–2.689	0.0001
BC011634_1	G protein-coupled receptor 30, GPR30	–3.429	0.0004	–2.138	0.0420
BC021104_1	Apelin	–2.024	0.0011	–2.162	0.0280
M55513_1	Potassium channel protein	–2.396	0.0001	–3.810	0.0007
NM_016279_1	E-cadherin	–2.197	0.0001	–2.048	0.0416
NM_145752_1	CDP-diacylglycerol--inositol 3-phosphatidyl-transferase isoform 2	–3.327	0.0002	–2.854	0.0001
		FTA		PTC	
BC002530_1	ADP-ribosylation factor-like 2, ARL2	–2.016	<0.05	–2.525	0.0086
NM_000946_1	Primase, polypeptide 1 (49kDa); PRIM1	–2.160	<0.05	–3.368	0.0004
NM_001559_1	Interleukin 12 receptor, beta 2, IL12RB2	–3.000	<0.05	–2.593	0.0087
NM_002076_1	Glucosamine (*N*-acetyl)-6-sulfatase precursor	–2.567	<0.05	–3.140	0.0003
NM_006298_1	Zinc finger protein 192, ZNF192	–2.003	<0.05	–2.037	0.0411
NM_006484_1	Dual-specificity tyrosine-(y)-phosphorylation regulated kinase 1b isoform c	–2.050	<0.05	–2.870	0.0008
XM_007868_1	Sodium/hydrogen exchanger 5 (Na(+)/H(+) exchanger 5),NHE-5	–2.655	<0.05	–2.644	0.0071
		FTC		PTC	
AF052510_1	Phosphocholine citidylil transferase b	–2.562	0.0001	–2.123	0.0156
AF107044_1	DNA-binding protein, SOX21	–2.488	0.0010	–2.876	0.0075
AK091478_1	NIL-2-a zinc finger protein	–2.260	0.0004	–2.090	0.0051
NM_002662_1	Phospholipase d1, phophatidylcholine-specific; PLD1	–2.001	0.0001	–2.742	0.0005
NM_002753_1	Mitogen-activated protein kinase 10, isoform 1; MAPK10	–2.277	0.0002	–2.214	0.0092
NM_003691_1	Serine/threonine kinase 16, STK16	–2.250	0.0010	–3.074	0.0033
NM_005251_1	Forkhead box C2, FOXC2	–2.351	0.0012	–3.124	0.0001

Table III. *Genes overlapping in underexpression FTA: follicular thyroid adenoma, FTC: follicular thyroid carcinoma, PTC: papillary thyroid carcinoma.*

		FTA		FTC		PTC	
ID	Name	Log. ratio	*p*-value	Log. ratio	*p*-value	Log. ratio	*p*-value
AF140630_1	Urotensin-II	–2.264	0.004	–4.106	0.001	–2.460	0.001
AF294009_1	Eosinophil-derived neurotoxin, RNS2	–2.113	0.024	–2.872	0.001	–2.501	0.014
AF308819_1	Nuclear receptor-interacting factor, PPARγ	–2.647	0.001	–2.418	0.029	–2.371	0.001

significantly modulated candidate genes, but for a set of genes acting on similar antiapoptotic and signalling pathways, and for those showing overlaps between the early-stage epithelial thyroid tumour types. Although the role of the overlapping genes between the histologically different tumour types with a common epithelial origin has not been clarified, they might represent early or pivotal steps of deregulation in tumorigenesis.

In this investigation follicular tumour types exhibited the down-regulation of *BTK, CDP diacylglycerol, GPR30* and *E-cadherin*. *BTK* and *CDP diacylglycerol* act on the same inositol-phospholipid signal transducing pathway, responsible for activating protein kinase C. *BTK* was previously demonstrated to be a mediator of oxidative stress induced apoptosis. *BTK* deficient B cells showed high resistance to oxidative stress-induced apoptosis (6). We found *GPR 30* underexpressed both in follicular adenoma and follicular carcinoma and expression-ratios were higher in adenoma tissues. *GPR 30* binds estrogen with high affinity and resides in the endoplasmatic reticulum, where it activates multiple

intracellular signalling (7). *GPR 30* and *MAPK* pathway were previously found to mediate the growth response to estrogens (17 beta esrtadiol, genistein and 4 hydroxytamoxifen) that could induce the progression of thyroid carcinogenesis (8). We recognized down-regulation of *E-cadherin* which was characteristic for both follicular tumour types (follicular adenoma and follicular carcinoma). *E-cadherin* is a cellular adhesion molecule mediating Ca^{2+}-dependent homophilic cell-cell adhesion thus playing important role in the maintenance of normal tissue architecture (9) and its down-regulation among thyroid carcinomas has already been demonstrated (10). In a real-time quantitative analysis of *E-cadherin* expression in thyroid neoplasms carried out by Smyth *et al.* follicular thyroid carcinomas consistently had significantly decreased *E-cadherin* expression compared with papillary thyroid carcinomas (11).

The most remarkable overlapping gene between follicular adenoma and papillary carcinoma was the interleukin *12 receptor β-2 (IL12Rβ-2)*. The *IL12Rβ-2* gene has previously found to be silenced in tumour cells from different human B-cell malignancies. It drives T helper responses, enhances T and NK cell cytotoxicity, and induces interferon-γ production by T and NK cells. In addition, IL-12 exerts antitumour activity through interferon-γ-dependent mechanisms (12).

We found the underexpressions *phospholipase D1 (PLD1)* and *mitogen-activated protein kinase 10 (MAPK10)* overlapping in the malignant histiotypes. These genes have previously been described and investigated in thyroid neoplastic models. *MAPK10* was identified in human thyroid cells and tissue and in PCR-validated homozygous deletions it found to be a candidate tumour suppressor gene as a target specific transcriptional factor inducing apoptosis and generating tissue specific responses (13, 14). The underexpression of *PLD1* seems to be contradictory at first sight as it is a downstream target of the *RAS*/*RalA GTPase* cascade implicated in mitogenic and oncogenic signalling contributing to the progression to a malignant phenotype in cells. Its expression is regulated by *PKC*, ADP ribosylation factors and Rho family proteins affecting signal transduction, vesicular trafficking and cytoskeletal reorganization, implicated in the metastatic potential of malignancies (15, 16). We identified *PLD1* down-regulation both in follicular carcinoma and papillary carcinoma. A possible reason for this result might be the early developmental stage of the tumour samples. On clinical and histopathological evaluation, neither of the tumour sample types showed metastatic features either locally or systemically.

There were some novel genes among the consequently down-regulated ones that we identified in the groups of follicular and papillary thyroid carcinoma, which had not been reported yet in thyroid malignancies, such as *SOX21* and *NIL-2-A*. Members of the SOX family are transcription factors required for development and differentiation of multiple cell lineages (17). *NIL-2-A* encodes a zinc finger protein inhibiting T lymphocyte specific interleukin-2 gene expression (18). The role of these genes in thyroid neoplasms has not been investigated yet.

We found a consequent down-regulation of three particular genes in all investigated tumour histiotypes: *Peroxisome proliferator-activated receptor-gamma (PPARγ), eosinophil-derived neurotoxin and urotensin II*. *PPARγ* is a member of a nuclear hormone receptor subfamily of transcription factors and found to be a promoter-specific repressor of *nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB)* (19). In a mouse model for thyroid carcinogenesis, Kato Y *et al.* found that reduced *PPARγ* protein abundance led to the activation of NF-κB signalling pathway and resulted in repression of critical genes involved in apoptosis and promotion of follicular thyroid carcinogenesis (20). There is also evidence showing that the *PPARγ* transcriptional pathway can include terminal differentiation of malignant breast epithelial cells and prostate adenocarcinoma cells (21). Eosinophil-derived neurotoxin is a cytosolic ribonuclease exhibiting tumour cell toxicities and telomerase degradative activity in Kaposi's sarcoma and Hodgkin's lymphoma and found to be useful for targeted therapy in both cancer types (22-23). The role of *urotensin II* in carcinogenesis is unclear. In a study UII-receptor activation was functionally coupled to phospholipase C-mediated Ca^{2+} mobilization (24).

Among the few constitutively up-regulated genes found in this investigation, some characteristic ones such as *cyclin D1 (CYLD1)*, were represented in papillary carcinoma, and *EDA1* in follicular adenoma. The *CYLD1* gene has previously been identified in thyroid carcinogenesis in both papillary (25) and follicular thyroid carcinoma (26) with excellent specificity, furthermore its regulatory role on *NF-κB* signalling has already been recognised and currently researched. *EDA1* was found to be a member of the TNF-related ligand family regulating epithelial development (27). It activates *NF-κB*-promoted transcription and has a regulatory role in *JNK* (including *MAPK10*) pathways (28). Among the overexpressed genes we found just one to be significantly regulated in all follicular tumour types: *NF-κB*. *NF-κB* belongs to the category of rapidly acting primary transcriptional factors, acting as a first responder to a wide variety of cell surface receptor stimuli regulating genes that control cell proliferation and cell survival. In most cell types, *NF-κB* is retained in the cytoplasm in its inactive form. A wide variety of external stimuli (including TNF, IL-1, PMA, H_2O_2, endotoxin and hypoxia) can induce its activation. Most of these agents induce NF-κB to translocate to the nucleus, where it activates transcription of target genes partaking in the suppression of apoptosis. Its activity was constitutively elevated in primary human thyroid carcinomas and correlated malignant phenotype (29-31).

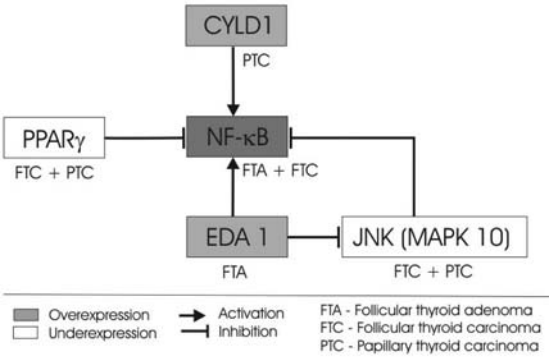

Figure 2. *Gene interactions of NF-κB found in thyroid tumours.*

Activated *NF-κB* was also detected in an *in vitro* model of human thyroid cancer that resembles the *in vivo* differentiated and undifferentiated thyroid tumours. In these cell lines persistent *NF-κB* activity was progressively detected in papillary thyroid carcinoma cells to follicular carcinoma cells until reaching the highest levels in anaplastic carcinoma cells reflecting the sustained activation of *NF-κB* confers an advantage for clonal selectivity (32).

All the histiotypes of tumours investigated here contained significantly modulated genes acting on the *NF-κB* regulatory pathway (Figure 2). In follicular histiotypes (follicular adenoma and follicular carcinoma) *NF-κB* was found to be up-regulated. Follicular thyroid adenomas exhibited the overexpression of *EDA1*, wich has a regulatory role in *NF-κB*-promoted transcription and *JNK* signalling. Malignant phenotypes (follicular thyroid carcinoma and papillary thyroid carcinoma) additionally represented constitutive down-regulation of genes (*PPARγ* and *MAPK10*/*JNK*) partaking in *NF-κB* inhibition. Furthermore in papillary thyroid carcinoma another regulatory gene (*CYLD1*) was consequently up-regulated, which is closely connected to *NF-κB* signalling. Our findings support the previous assumptions that modulation of *NF-κB* signal transduction plays a crucial role in early thyroid carcinogenesis and further investigations could possibly open the door to diagnostic, therapeutic or preventative perspectives.

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