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Metallothionein Isoform Expression in Benign and Malignant Thyroid Lesions

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Abstract. *Background: Metallothioneins (MTs) are involved in numerous cell processes such as binding and transport of zinc and copper ions, differentiation, proliferation and apoptosis, therefore contributing to carcinogenesis. Scarce data exist on their expression in benign and malignant lesions of the thyroid. Materials and Methods: mRNA expression of functional isoforms of MT genes (MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1X, MT2A, MT4) was studied in 17 nodular goiters (NG), 12 follicular adenomas (FA) and 26 papillary thyroid carcinomas (PTC). Results: One-way ANOVA revealed significant differences in mRNA expression levels of MT1A ($p<0.05$), MT1E ($p<0.005$), MT1F ($p<0.0001$), MT1G ($p<0.005$), MT1X ($p<0.0005$) and MT2A ($p<0.005$) in the analyzed samples. Post hoc analysis confirmed a significantly lower expression of MT1A mRNA in PTC compared to NG ($p<0.05$). Significant down-regulation was also noted for other MT isoforms in PTC in comparison to NG: MT1E ($p<0.05$), MT1F ($p<0.0001$), MT1G ($p<0.005$), MT1X ($p<0.0005$) and MT2A ($p<0.05$). In addition, significant down-regulation of MT1F and MT1G in FA compared to NG was observed ($p<0.005$ and $p<0.05$, respectively). Conclusion: Expression of functional MT isoforms may contribute to thyroid carcinogenesis and potentially serve as a diagnostic marker in distinguishing benign and malignant lesions.*

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Nodular goiter (NG) is one of the most common iodine intake-dependent endocrine disorders, as thyroid nodules can be palpated in approximately 4-7% of adults (1). With advancements of ultrasound technique and more widespread access to imaging, incidental diagnoses of thyroid nodules have increased to include up to 20% of the general population (2). The incidence rate of thyroid neoplasms in Poland has grown substantially in the past two decades and was 2,192 cases in 2010. This incidence rate increases with age up to the seventh decade of life, and decreases thereafter (3).

It is of great importance to distinguish benign from malignant lesions before surgical treatment because thyroid operations are associated with dangerous complications such as recurrent laryngeal nerve palsy and hypoparathyroidism. Fine-needle aspiration (FNA) biopsy of the thyroid is routinely used in preoperative diagnostic examination for thyroid nodules (4). However, FNA biopsy has certain limitations, as up to 40% of cases are diagnosed as of indeterminate malignancy (follicular neoplasms) (5, 6). Firstly, there is no possibility of differentiating a follicular adenoma (FA) from a follicular thyroid carcinoma (FTC) upon cytological assessment. In order to diagnose FTC, it is necessary to assess capsule infiltration and vascular invasion during histopathological examination (7). All patients with indeterminate cytology undergo surgical treatment, with only 10-20% ultimately having a diagnosis of malignant disease, meaning 80-90% could have avoided surgical treatment if an appropriate marker were available. Secondly, it is very difficult to effectively control all nodular lesions in multinodular goiter, as very often papillary thyroid carcinoma (PTC) are diagnosed in histopathological examination (8). The overall incidence of thyroid carcinomas in those with potentially benign thyroid lesions is up to 17.2% of resected cases, however, incidental thyroid carcinomas are prevalent in up to 35.6% upon autopsy examination (9, 10).

Therefore, identification of potential markers of malignancy in thyroid nodules in the form of single or multiple molecular targets is of upmost importance. Metallothioneins (MTs) may be potentially useful markers in this clinical setting. Metallothioneins are intracellular low molecular weight proteins (6-7 kDa) first isolated in 1957 from horse renal cortex, and are expressed both in normal as well in neoplastic cells (11, 12). All MT isoforms posses a highly conserved amino acid sequence and show minimal structural differences. A single MT molecule consists of 61-68 amino acids, depending on the isoform, where up to one-third of the protein sequence is composed of cysteine residues (13-15). Two domains are distinguished, the C-terminal α and the N-terminal β, which are linked each other by a lysine dimer. MTs are divided into four principal isoforms, MT1, MT2, MT3 and MT4 (16). At least 11 MT genes code for functional proteins namely *MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1M, MT1X, MT2A, MT3* and *MT4* (16-18). *MT1C, MT1D, MT1I, MT1J, MT1K, MT1L* and *MT2B* are non-coding pseudogenes with unknown functionality (14, 16, 19).

Due to their high cysteine content, MTs are involved in numerous cell processes such as binding and transport of zinc and copper ions, detoxification of heavy metals, scavenging of free radicals, and cell differentiation, proliferation and apoptosis (14, 20, 21). Furthermore, MT1, MT2 and MT3 may protect cells from certain chemotherapeutic agents or radiation-induced damage (22, 23). Several studies reported increased expression of MTs in cancer such as of breast, kidney, lung, nosopharynx, ovary, prostate and salivary glands (13, 20).

The first study regarding MT expression in normal thyroid tissues was performed by Nartney *et al*. in the late 1980s. They revealed that in paraffin-embedded tissues of normal thyroid, only 20% of analyzed cases expressed MT in the nucleus of follicular thyroid cells, whereas a nuclear-cytoplasmic expression pattern was noted in majority of surgically resected tumor samples (91%) (24). Nevertheless, other reports indicate that MT expression is highly variable dependent on the particular lesion type, but in most cases it is down-regulated in thyroid cancer as compared to normal and benign thyroid lesions (25-28). In the study of Królicka *et al*., the highest expression of MT1 and 2 was detected in FTC and the lowest in medullary carcinoma. Its expression was also significantly elevated in FTC as compared to FA and may be potentially utilized as a biomarker of distinguishing both these lesions (25). Moreover, Ferrario *et al*. identified the MT1G isoform as a potent oncosuppressor of thyroid carcinogenesis (26).

Taking into account the biological role of MT and up until now performed studies we investigated the mRNA expression functional MT isoforms genes (*MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1X, MT2A, MT4*) in benign and malignant thyroid tissues in order to assess their potency in discriminating between these lesion types.

Table I. *Clinical and pathological characteristics of the analyzed patients.*

Characteristic	Nodular goiter	Follicular adenoma	Papillary thyroid carcinoma*
Age (range), years	49.59±14.31 (27-81)	49.25±17.65 (23-87)	54.23±14.31 (27-81)
Gender, n			
Male	1	2	5
Female	16	10	21
pT, n			
pT1			10
pT2			5
pT3			1
pT4			6
pN, n			
pN-			15
pN+			7

*pT and pN characteristics missing in four cases.

Materials and Methods

Patients and clinical samples. The studies were performed on thyroid tissue samples collected during thyroidectomy procedures at the First Department of General, Gastroenterological and Endocrinological Surgery of Wroclaw Medical University, Poland, in the years 2009-2011. The study group comprised 17 NG, 12 FA and 26 PTC. The clinical and pathological data of the patients are summarized in Table I.

The resected tissue fragments were divided into two parts. One was fixed in 10% buffered formalin, dehydrated, embedded in paraffin and subsequently hematoxylin and eosin (HE) staining was performed to verify the diagnosis, and qualify the sample for the molecular studies. The second was collected in RNAlater (Invitrogen, Carlsbad, CA, USA) and stored at −80˚C until RNA isolation was performed.

RNA extraction, cDNA synthesis and real-time polymerase chain reaction (PCR). Total RNA was extracted from thyroid samples stored in RNAlater using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the procedures recommended by the manufacturer. DNase (Qiagen) digestion was performed to remove genomic DNA. The quality of the RNA samples was evaluated utilizing agarose gels and ethidium bromide staining for visualization of *18S* and *28S* bands under UV light. Concentration and quality of the isolated RNA was measured in a NanoDrop1000 instrument (NanoDrop Technologies, Wilmington, MA, USA). Reverse transcription was performed using SuperScript III (Invitrogen) according to the manufacturer's protocol.

Subsequently, mRNA expression levels of the functional MT isoforms were evaluated with use of real-time PCR, performed in a 7900HT Fast Real-Time PCR System using the TaqMan® Gene

Table II. *Relative mRNA expression of the analyzed metallothionein (MT) isoforms. Differences in expression were analyzed using one-way ANOVA. Data are presented as the mean±standard deviation (median).*

MT isoform	Nodular goiter	Follicular adenoma	Papillary thyroid carcinoma	*p*-Value
MT1A	1.42±1.30 (1.13)	1.09±1.12 (0.44)	0.53±0.43 (0.39)	<0.05
MT1B	43.45±149.1 (0.34)	16.89±39.15 (0.09)	4.11±11.81 (0.13)	ns
MT1E	1.59±1.47 (1.21)	0.32±0.36 (0.23)	0.66±1.18 (0.22)	<0.005
MT1F	1.49±1.29 (0.85)	0.32±0.42 (0.11)	0.29±0.55 (0.07)	<0.0001
MT1G	2.33±3.02 (1.60)	0.43±0.51 (0.30)	0.40±1.24 (0.04)	<0.005
MT1H	8.97±20.58 (2.53)	2.95±8.49 (0.19)	0.92±4.19 (0.01)	ns
MT1X	1.14±0.75 (1.01)	0.60±0.48 (0.58)	0.35±0.41 (0.18)	<0.0005
MT2A	1.49±0.88 (1.27)	0.73±0.64 (0.61)	0.81±0.72 (0.61)	<0.05
MT3	8.35±20.04 (0.93)	68.08±187.9 (3.86)	0.48±0.26 (0.45)	ns

ns: Not significant.

Table III. *Correlations between mRNA expression of analyzed functional metallothionein (MT) isoforms in nodular goiter cases.*

Isoform	*MT1B*	*MT1E*	*MT1F*	*MT1G*	*MT1H*	*MT1X*	*MT2A*	*MT3*
MT1A	r=0.46	r=0.43	r=0.53	r=0.36	r=0.40	r=0.44	r=0.42	r=0.04
	ns	ns	*p*<0.05	ns	ns	ns	ns	ns
MT1B		r=0.70	r=0.49	r=0.62	r=0.65	r=0.65	r=0.49	r=0.07
		p<0.005	*p*<0.05	*p*<0.005	*p*<0.005	*p*<0.005	*p*<0.05	ns
MT1E			r=0.65	r=0.40	r=0.34	r=0.81	r=0.73	r=–0.24
			p<0.005	ns	ns	*p*<0.0001	*p*<0.005	ns
MT1F				r=0.73	r=0.70	r=0.62	r=0.72	r=0.22
				p<0.005	*p*<0.005	*p*<0.05	*p*<0.005	ns
MT1G					r=0.82	r=0.59	r=0.65	r=0.59
					p<0.0001	*p*<0.05	*p*<0.005	*p*<0.05
MT1H						r=0.40	r=0.43	r=0.46
						ns	*p*<0.05	ns
MT1X							r=0.78	r=0.13
							p<0.0001	ns
MT2A								r=0.03
								ns

ns: Not significant.

Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA). The results of mRNA expression were normalized to the mRNA expression of β-actin. For the PCR reactions, the following TaqMan® primers and probes utilized in our previous studies were used: *MT1A* Hs00831826_s1, *MT1B* Hs01875377_s1, *MT1E* Hs01938284_g1, *MT1F* Hs00744661_sH, *MT1G* Hs02578922_gH, *MT1H* Hs00823168_g1, *MT1X* Hs04194245_g1, *MT2A* Hs01591333_g1, *MT3* Hs00359394_g1, *MT4* Hs00262914_m1, *ACTB* Hs99999903_m1 (Applied Biosystem) (29, 30). All of the reactions were performed in triplicates. Polymerase was activated at 50˚C for 2 min. Initial denaturation was performed at 94˚C for 10 min followed by 40 cycles of denaturation at 94˚C for 15 s, an annealing step and synthesis at 60˚C for 1 min. Relative expression (RQ) of the studied MT isoforms was calculated using the ΔΔCt method. The mean expression of MT isoforms of the 17 NG cases was used as calibrator for MT isoform expression in all the analyzed samples.

Statistical analysis. Prism 5.0 (GraphPad, La Jolla, CA, USA) statistical software was used for statistical analysis. One-way analysis of variance (ANOVA) was utilized to determine significant differences in mRNA RQs for MT isoforms in all the analyzed groups. The Bonferroni multiple comparison test was subsequently used to adjust the obtained results for multiple testing corrections and determine the differences of MT mRNA expression levels between two particular groups. Correlations between the relative expression (RQ) of MT mRNAs were analyzed using Spearman correlation test, whereas correlation of expression with patient age was performed utilizing the Pearson correlation test. In all the analyses, results were considered statistically significant when $p<0.05$.

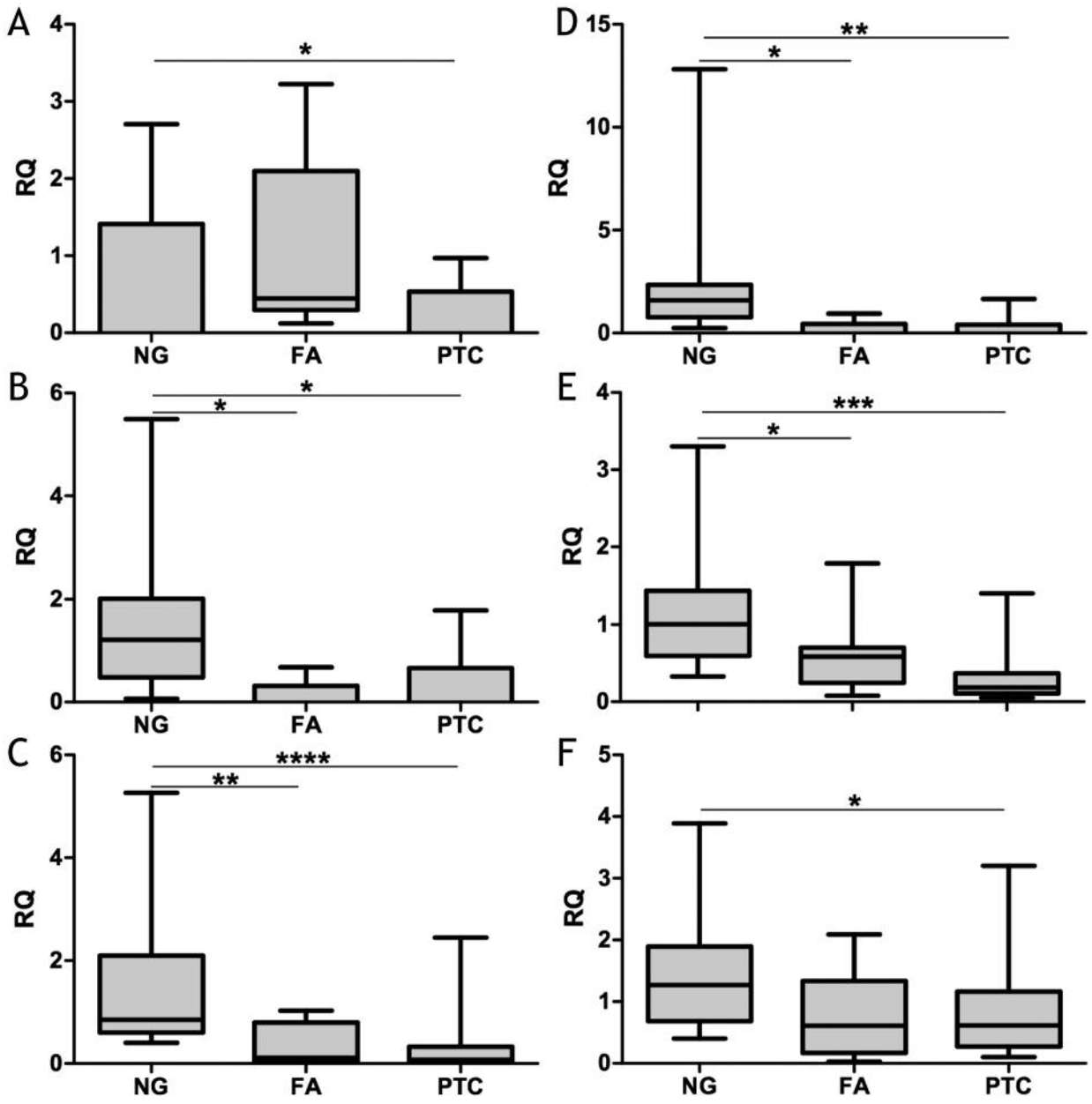

Figure 1. *Relative expression (RQ) of metallothionein (MTs) MT1A (A), MT1E (B), MT1F (C), MT1G (D), MT1X (E) and MT2A (F) in nodular goiter (NG), follicular adenoma (FA), and papillary thyroid cancer (PTC). Data are presented as box-plots. Significantly different at *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001; Bonferroni multiple comparison test.*

Results

Relative expression of analyzed MT isoforms is summarized in Table II, however, no *MT4* mRNA expression was detected in any of the analyzed samples. One-way ANOVA analysis revealed significant differences in mRNA RQs of *MT1A* ($p<0.05$), *MT1E* ($p<0.005$), *MT1F* ($p<0.0001$), *MT1G* ($p<0.005$), *MT1X* ($p<0.0005$) and *MT2A* ($p<0.005$) within analyzed samples. No significant differences were noted in mRNA RQs of *MT1B, MT1H* and *MT3*.

Post hoc analysis using Bonferroni multiple comparison test revealed significant differences in the particular MT isoforms RQs in the study group. A significantly lower expression of *MT1A* mRNA was observed in PTC compared

Table IV. *Correlation between mRNA expression of analyzed functional metallothionein (MT) isoforms in follicular adenoma cases.*

Isoform	*MT1B*	*MT1E*	*MT1F*	*MT1G*	*MT1H*	*MT1X*	*MT2A*	*MT3*
MT1A	r=0.50 ns	r=0.56 ns	r=0.32 ns	r=0.28 ns	r=0.53 ns	r=0.18 ns	r=0.20 ns	r=0.02 ns
MT1B		r=0.35 ns	r=–0.10 ns	r=–0.23 ns	r=0.28 ns	r=0.23 ns	r=–0.08 ns	r=0.24 ns
MT1E			r=0.60 $p<0.05$	r=0.37 ns	r=0.36 ns	r=0.54 ns	r=0.59 $p<0.05$	r=–0.36 ns
MT1F				r=0.92 $p<0.0001$	r=0.64 $p<0.05$	r=0.85 $p<0.05$	r=0.85 $p<0.0001$	r=–0.27 ns
MT1G					r=0.66 $p<0.05$	r=0.69 $p<0.05$	r=0.85 $p<0.005$	r=–0.19 ns
MT1H						r=0.58 $p<0.05$	r=0.40 ns	r=0.32 ns
MT1X							r=0.69 $p<0.05$	r=–0.14 ns
MT2A								r=–0.34 ns

ns: Not significant.

Table V. *Correlation between mRNA expression level of analyzed functional metallothionein (MT) isoforms in papillary thyroid carcinoma cases.*

Isoform	*MT1A*	*MT1B*	*MT1E*	*MT1F*	*MT1G*	*MT1H*	*MT1X*	*MT2A*	*MT3*
MT1A		r=0.36 ns	r=0.63 $p<0.0001$	r=0.57 $p<0.0005$	r=0.50 $p<0.05$	r=0.29 ns	r=0.48 $p<0.05$	r=0.46 $p<0.05$	r=0.26 ns
MT1B			r=0.15 ns	r=0.31 ns	r=0.26 ns	r=0.49 $p<0.05$	r=0.07 ns	r=0.29 ns	r=–0.29 ns
MT1E				r=0.65 $p<0.0001$	r=0.64 $p<0.0001$	r=0.41 $p<0.05$	r=0.69 $p<0.0001$	r=0.59 $p<0.005$	r=0.23 ns
MT1F					r=0.93 $p<0.0001$	r=0.75 $p<0.0001$	r=0.75 $p<0.0001$	r=0.54 $p<0.05$	r=0.17 ns
MT1G						r=0.80 $p<0.0001$	r=0.76 $p<0.0001$	r=0.52 $p<0.05$	r=0.34 ns
MT1H							r=0.60 $p<0.005$	r=0.34 ns	r=0.18 ns
MT1X								r=0.75 $p<0.0001$	r=0.23 ns
MT2A									r=–0.05 ns

ns: Not significant.

to NG ($p<0.05$). Significant down-regulation was also noted for other MT isoforms in PTC in comparison to NG: *MT1E* ($p<0.05$), *MT1F* ($p<0.0001$), *MT1G* ($p<0.005$), *MT1X* ($p<0.0005$) and *MT2A* ($p<0.05$). In addition, significant down-regulation of *MT1F* and *MT1G* in FA as compared to NG was observed ($p<0.005$ and $p<0.05$, respectively) (Figure 1).

Statistical analysis using the Mann–Whitney test revealed no significant associations between mRNA expression levels and primary tumor size or presence of lymph node metastases (data not shown). Moreover, no significant correlations were found with patient age at diagnosis and mRNA RQs of particular MT isoforms (Pearson correlation test) in benign lesions (NG, FA) and PTC (data not shown). Spearman correlation test revealed significant correlations of mRNA expressions of analyzed in MT isoforms in benign and malignant thyroid lesions (Tables III-V).

Discussion

It is very difficult to effectively control all nodular lesions in multinodular goiter, as PTCs are very often diagnosed upon histopathological examination and detected in up to 35.6% of potentially benign thyroid glands upon post-mortem autopsy (10). Therefore, it is important to identify new markers based on ancillary techniques *e.g.* mRNA expression, which could improve the differentiation of benign from malignant thyroid lesions during FNA examination. It was already shown that expression of MT isoforms among thyroid tumor types differs significantly. Down-regulation of *MT1E, MT1G, MT1X* and *MT2A* in PTC in comparison to benign thyroid lesions was shown by Ferrario *et al.* (26) and Huang *et al.* (28). The results of this study confirm the observations of other authors, however, we additionally demonstrated significantly lower expression of the *MT1A* and *MT1F* in PTC. No significant differences between expression of the *MT1B, MT1H* and *MT3* in the analyzed cases were shown. Although the immunohistochemical study of Nartey *et al.* demonstrated higher MT expression in PTC (24), the recently published study by Królicka *et al.* showed lower MT1/2 protein expression in PTC as compared to NG (25). Our results support the latter finding.

The potential mechanism underlying the differences in mRNA expression of MT isoforms between the analyzed lesions have not yet been determined, however, the redox potential of thyroid cells during malignant transformation may impact the level of MT expression due to the redox sensitive nature of regulatory mechanism (14, 20). The observed strong positive correlations between mRNA expression of particular MT isoforms seem to support this hypothesis.

Nevertheless based on the obtained results, determination of MT mRNA expression could be useful in identification of cancerous nodules within NG, as *MT1A, MT1E, MT1F, MT1G, MT1X* and *MT2A* were significantly down-regulated in PTCs. It seems that determination of single MT isoforms would not be sufficient to discriminate benign and malignant lesions as significant down-regulation of *MT1E, MT1F* and *MT1X* was also be noted in FA samples compared to NG and did not significantly differ from the levels determined in PTCs. Therefore, it seems that combined determination of *MT1A, MT1E, MT1F, MT1G, MT1X* and *MT2A* would be able to distinguish the underlying malignancy within the NG structure and also combined with cytological assessment of FNA could increase the detection of malignant changes.

However, it should be underscored that the results of this study are preliminary and of limited potential as determination of MT mRNA levels was performed in tissues biopsied following surgical removal of thyroid lesions. In order to fully validate the potential utility of our preliminary findings, further studies undertaken on FNA of thyroid lesions are necessary to determine the clinical significance of presented results. In summary, expressions of functional MT gene isoforms may be potential markers of malignant transformation of thyroid tissues and therefore should be of clinical relevance in the diagnostic procedures of this endocrine gland.

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