

## Comparison of Metallothionein (MT) and Ki-67 Antigen Expression in Benign and Malignant Thyroid Tumours

ANNA KRÓLICKA<sup>1</sup>, CHRISTOPHER KOBIERZYCKI<sup>1</sup>, BARTOSZ PUŁA<sup>1</sup>, MARZENA PODHORSKA-OKOŁÓW<sup>1</sup>,  
ALEKSANDRA PIOTROWSKA<sup>1</sup>, MARTA RZESZUTKO<sup>2</sup>, WOJCIECH RZESZUTKO<sup>2</sup>, JERZY RABCZYŃSKI<sup>2</sup>,  
PAWEŁ DOMOSŁAWSKI<sup>3</sup>, BEATA WOJTCZAK<sup>3</sup>, JANUSZ DAWISKIBA<sup>3</sup> and PIOTR DZIĘGIEL<sup>1,4,5</sup>

Departments of <sup>1</sup>Histology and Embryology, <sup>2</sup>Pathomorphology and <sup>3</sup>Gastroenterological and Endocrinological  
Surgery, Medical University in Wrocław, Poland;

<sup>4</sup>Department of Histology and Embryology, Medical University of Poznan, Poznan, Poland;

<sup>5</sup>Lower Silesia Centre of Oncology, Wrocław, Poland

**Abstract.** *Thyroid carcinomas are the most frequently occurring tumours in the endocrine system. Metallothioneins (MT) and Ki-67 proteins are present in intensely proliferating cells, and their expression has been observed in numerous tumours, including thyroid tumours. The purpose of this study was to analyse the relationship between intensity of MT expression and Ki-67 antigen on one hand and histological features of the examined thyroid tumours on the other. The investigated material included 186 archival paraffin blocks with samples of various thyroid tissues, obtained from the Chair and Department of Pathomorphology, Medical University of Wrocław. In paraffin sections, immunohistochemical reactions were performed with the use of monoclonal anti-MT (I/II) and anti-Ki-67 antibodies. Intensity of MT and Ki-67 antigen expression was evaluated using a light microscope using the semi-quantitative method of Remmele. A significant difference in MT expression was noted between different tumours of the thyroid: the highest expression was detected in follicular carcinoma and the lowest was detected in medullary carcinoma. Expression of MT was also significantly elevated in follicular carcinoma as compared to follicular adenoma. On the other hand, no significant differences were seen between expression of Ki-67 antigen in follicular adenoma and follicular carcinoma. Moreover, these investigations detected no correlation between the expression of MT and Ki-67 antigen in follicular adenoma and follicular carcinoma. In view of the obtained results, the*

*expression of MT can be considered as a potential marker of differentiation between the two types of thyroid tumours, which are otherwise difficult to differentiate.*

Carcinomas of the endocrine glands and organs are manifested relatively infrequently. Tumours of the thyroid gland are noted most frequently, comprising 1.8% out of 1.28 million new cases of extradermal cancer, which resulted in 0.41% of 555,500 deaths due to these tumours in the United States in 2002 (1). The highly differentiated carcinomas (papillary and follicular carcinomas) develop from cells of thyroid follicles and they develop most frequently in young, while poorly differentiated anaplastic carcinomas are more frequent in people over 60 years of age. The papillary carcinoma accounts for 70-80% of thyroid tumours and is regarded to be the least aggressive form of carcinoma of the thyroid (2). In turn, follicular carcinoma accounts for 5-15% cases of cancer in the thyroid. Due to difficulties encountered in its differentiation from follicular adenoma, a benign lesion, the two types of tumours are described as follicular tumours. Their differentiation can be executed exclusively in the post-surgical preparation, but the lesions cannot be distinguished using a targeted fine-needle aspiration biopsy (3). Other types of thyroid cancer, such as anaplastic carcinoma (5%) and medullary carcinoma (3-5%) are much less frequent and do not pose great difficulties in histopathological diagnosis (3).

Metallothioneins (MT) represent a family of low molecular weight proteins which are expressed in many species. They are highly conserved and are manifested in four principal isoforms of variable manifestation in either healthy or neoplastic tissues (4, 5). The polypeptide chain of MT consists of 61 to 68 amino acids (depending on the isoform) of which 30% of the residues involve cysteines. The MT molecule comprises two domains (C-terminal  $\alpha$  and N-terminal  $\beta$ ), linked with each other by a lysine dimer. The

Correspondence to: Piotr Dzięgiel, MD, Ph.D., Katedra i Zakład Histologii i Embriologii, Akademia Medyczna we Wrocławiu; ul. Chałubińskiego 6a, 50-368 Wrocław, Poland. Tel: +48 717841354, Fax: +48 717840082, e-mail: piotr@hist.am.wroc.pl

Key Words: Thyroid carcinoma, metallothionein, Ki-67.

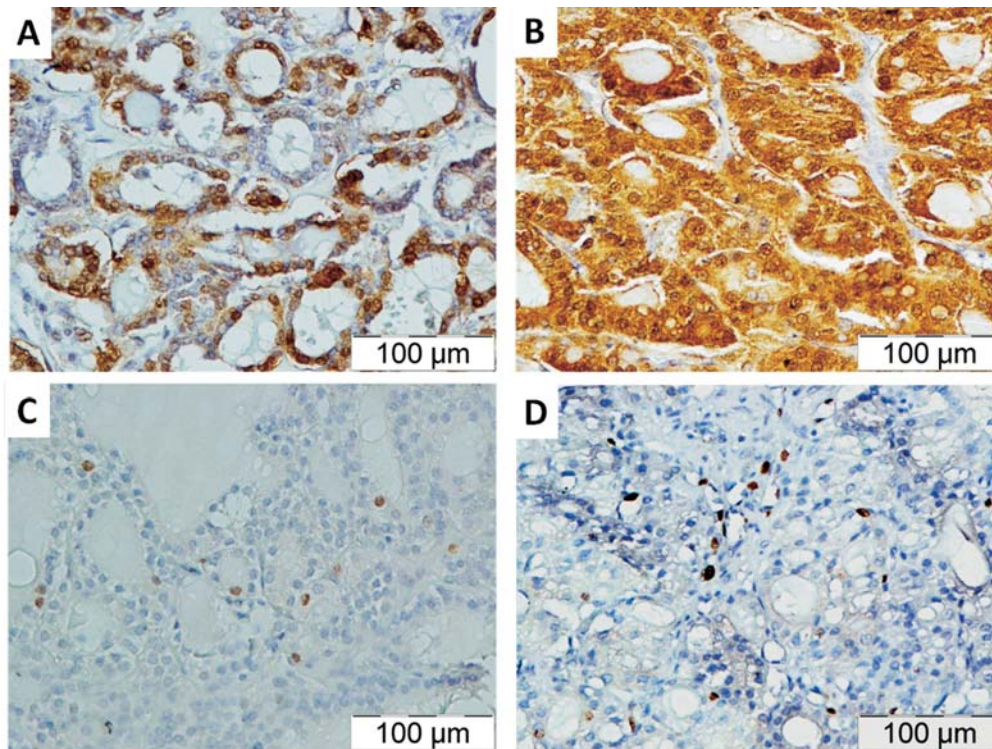


Figure 1. Nuclear/cytoplasmic expression of MT in (A) follicular adenoma and (B) follicular carcinoma and expression of Ki-67 antigen in (C) follicular adenoma and (D) follicular carcinoma.

physiological role of MT involves binding and transport of zinc and copper ions and detoxification by binding heavy metals, *e.g.* cadmium, mercury and lead (4, 5). In intensely dividing cells (*i.e.* neoplastic cells), an augmented expression of MT has been observed, which supplies zinc ions to enzymes participating in DNA replication and protects cells from apoptosis (5, 6). MT may affect the action of certain chemotherapeutic agents, by inactivating free radicals formed due to metabolism of certain cytostatic drugs, such as anthracyclins, and by binding to other agents, such as to cisplatin (7, 8). For more than ten years, studies have been conducted targeted at determining prognostic and diagnostic role of MT in various types of human malignant tumour (9-11).

Ki-67, which belongs to the group of most frequently used proliferation markers, is a non-histone protein consisting of two polypeptide chains and is expressed in the cell nucleus. Its expression can be detected in the G<sub>1</sub>, S and mitotic cell cycle phases and the intensity of its expression allows the determination of the proliferative activity of cells (12, 13). High expression of Ki-67 antigen has been detected in the mammary gland, lungs, prostate, soft tissues tumours and in brain, but its biological role remains incompletely understood (13-16).

This study aimed to compare MT and Ki-67 expression intensities in various benign and malignant tumours of the thyroid gland using immunohistochemical techniques.

## Materials and Methods

**Patients.** The studies were conducted on 186 paraffin blocks of various thyroid tumours obtained from the Department of Pathomorphology, Medical University of Wrocław, Poland. A total of 92 malignant tumour samples (including 48 papillary carcinomas, 35 follicular carcinomas, 9 medullary carcinomas) and 31 adenomas and 63 samples of thyroid nodular goitre were examined.

**Immunohistochemistry.** Tumour samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin blocks. All histochemical reactions (IHC) were performed in four µm thick paraffin sections, deparaffinised in xylene and rehydrated. In the cases of anti-Ki-67 IHC the sections were pre-incubated in a citrate buffer (pH 6, 10 mM) at the temperature of 95-98°C for 20 mins in order to reveal epitopes. Activity of endogenous peroxidase was blocked by five min incubation with 3% H<sub>2</sub>O<sub>2</sub>. Expression of MT I/II (clone E9) and of Ki-67 (clone MIB-1) was demonstrated using mouse monoclonal antibodies produced by DakoCytomation (Glostrup, Denmark). The antigens were visualised using biotinylated antibodies and streptavidin conjugated with horseradish peroxidase (DakoCytomation LSAB+ System-HRP). Diaminobenzidine (DAB, DakoCytomation) was used as the

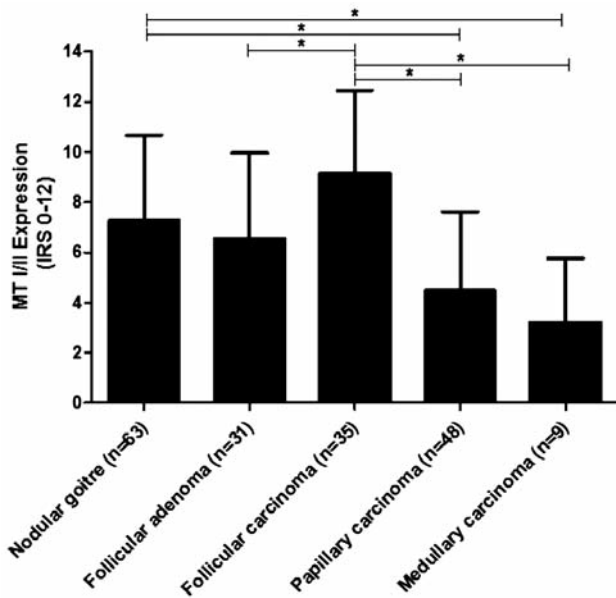


Figure 2. Intensity of MT expression in various types of thyroid lesions. A significant difference was detected between follicular carcinoma and, on the other hand, papillary carcinoma ( $p<0.0001$ ) or follicular adenoma ( $p=0.0011$ ). A significantly higher expression was also shown in follicular carcinoma as compared to medullary carcinoma ( $p<0.0001$ ). As compared to MT expression in nodular goitre, a significantly lower expression was noted in papillary carcinoma ( $p<0.0001$ ) and in medullary carcinoma ( $p=0.0021$ ). Asterisks indicate significant  $p$ -values.

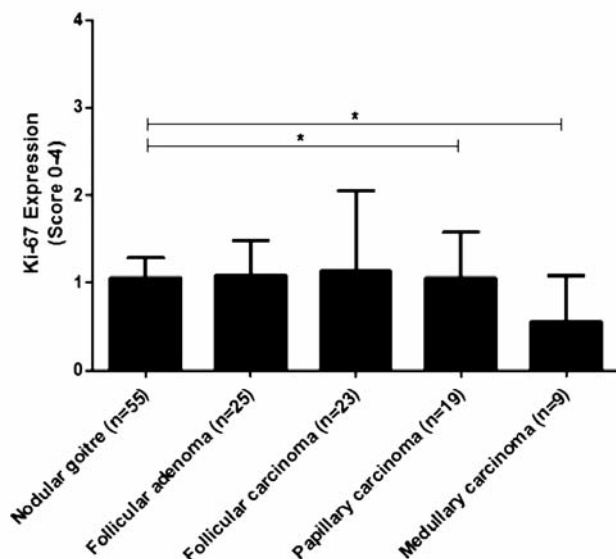


Figure 3. Intensity of Ki-67 antigen expression in various type of thyroid tumours. A significant difference in the expression was noted between medullary carcinoma and follicular adenoma ( $p<0.0057$ ) and between medullary carcinoma and nodular goitre ( $p<0.0001$ ). Asterisks indicate significant  $p$  values.

substrate. All the reactions were conducted using negative controls. All slides were counterstained with hematoxylin.

**Evaluation of IHC reaction intensity.** Evaluation of MT reaction intensity was conducted using the semi-quantitative IRS method of Remmele (17), which is based on the intensity of the colour reaction and percentage of positive neoplastic cells in the preparation. The scale ranges from 0-12 points (pts), where 0 denotes absence of reaction, 1-2 corresponds to poor reaction, 3-4 to moderate reaction and 6-12 to a pronounced reaction. The intensity of Ki-67 antigen expression in neoplastic cells was evaluated according to the percentage of positive cells among all the neoplastic cells, where 0 corresponds to absence of the reaction, 1 denotes 1-10% positive cells, 2 corresponds to 11-25% positive cells, 3 to 26-50% and 4 to >50% positive cells.

**Statistical analysis.** The obtained results were subjected to statistical analysis using Prism 5.0 software (GraphPad, CA, USA). The applied tests included Spearman's correlation test, Kruskal-Wallis test and Mann-Whitney  $U$ -test. Differences were statistically regarded significant at  $p<0.05$ .

## Results

Among the 186 thyroid tumour samples that were examined, the nuclear/cytoplasmic expression of MT was noted in 180 cases (96.7%) (Figure 1A, B). High MT expression (score 6-12) was demonstrated in 73% nodular goitre cases, 70.9% cases of follicular adenoma (Figure 1A), 45.8% cases of papillary carcinoma and 85.7% follicular carcinoma cases (Figure 1B). A high expression of MT was noted in 33% cases of medullary carcinoma.

The most pronounced expression of MT was observed in follicular carcinomas ( $9.14\pm3.32$ ), and a lower but still significant expression was observed in nodular goitre ( $7.25\pm3.41$ ), follicular adenoma ( $6.54\pm3.40$ ) with the lowest expression detected in papillary carcinoma ( $4.47\pm3.14$ ) and medullary carcinoma ( $3.22\pm2.54$ ). Significant differences were observed in MT expression between individual types of thyroid tumours (Figure 2).

A significant difference in MT expression intensity was detected between follicular carcinoma (Figure 1B) and follicular adenoma (Figure 1A), which are extremely difficult to differentiate by histopathological examination. A statistically significantly distinct expression was detected in papillary carcinoma and follicular carcinoma and medullary carcinoma. In the papillary carcinoma and medullary carcinoma a significantly lower expression was noted than that in nodular goitre.

Among the 131 lesions that were examined, nuclear expression of Ki-67 antigen was detected in 118 cases. The most pronounced expression (Figure 3) was noted in follicular carcinoma ( $1.13\pm0.92$ ), with a slightly lower expression in follicular adenoma ( $1.08\pm0.4$ ), papillary carcinoma ( $1.05\pm0.52$ ) and nodular goitre ( $1.05\pm0.22$ ), and the lowest expression in medullary carcinoma ( $0.56\pm0.53$ ).



No significant differences were found in the intensity of Ki-67 antigen expression between follicular adenoma (Figure 1C) and follicular carcinoma (Figure 1D). A significant difference in expression of the antigen was detected between medullary carcinoma and nodular goitre as well as between medullary carcinoma and follicular adenoma (Figure 3).

Expressions of MT and that of Ki-67 antigens in follicular adenoma and follicular carcinoma lesions manifested no correlation when examined by Spearman's test ( $r=0.12$ ;  $p>0.05$ ).

## Discussion

Expression of the MT I/II and Ki-67 antigens have been compared in various types of tumours in many previous studies. They have been examined in tumours of large intestine, adrenal cortex, oral cavity, prostate, mammary gland, ovary, in soft tissue sarcomas and non-small cell lung carcinomas (11-13, 18-23). In the majority of the studies, an evident correlation has been found between the expressions of MT and Ki-67 antigens (9, 11-13).

To date, studies on expression of MT and its isoforms in benign and malignant lesions of thyroid gland have given often contradictory results. Nartey *et al.* (24) demonstrated expression of the proteins in only 20% of normal thyroid glands and in 91% of neoplastic lesions in the organ. For comparison, in the current studies as many as 97% of thyroid tumours have been found to manifest expression of MT. These results are partially consistent with recently published data, which demonstrate a decreased expression of MT isoforms in thyroid papillary carcinoma as compared to benign lesions, although the current results showed an increased expression intensity of MT in follicular carcinoma (25-29). Ferrario *et al.* (26) demonstrated a decreased expression of functional MT isoforms (MT1E, MT1G, MT1F and MT2A) in papillary carcinoma and, on the basis of a cellular model, they pointed to the potential role of MT1G as a suppressor of the neoplastic process. In the papillary thyroid carcinoma, a decreased expression of MT1G takes place due to hypermethylation of the gene promoter (27). Investigations conducted with the use of DNA based microarrays have also demonstrated a decreased expression of 13 MT genes in this type of thyroid tumour (29). The observations are consistent with the earlier published results on smaller case numbers (28). Other experiments have also demonstrated a decreased inactivation of free radicals in thyroid papillary carcinomas, which may be explained by a lowered expression of MT in this malignancy (30, 31). Studies of Liu *et al.* (32) showed that any of eight functional MT isoforms may undergo expression under stimulatory effect of cadmium and activation of ERK1/2 kinases in cells of thyroid papillary carcinoma. The cells in which the isoforms manifested expression, manifested a shorter G<sub>1</sub> phase and a more rapid transit into S and M phases of the

cell cycle. MT isoforms are also induced in cells of thyroid anaplastic carcinoma under the effect of calcium ions, in which the augmented MT expression resulted in an abbreviated G<sub>1</sub> phase of the cycle (33).

As compared to the current investigations, previously conducted studies that demonstrated a decreased expression of MT isoforms in malignant lesions were conducted on lower numbers of cases. Studies of Liu *et al.* (32, 33) seem to confirm the current results. The highly variable expression of MT in individual types of thyroid tumours may point to a variable cellular metabolism and their pronounced expression in thyroid follicular carcinoma may be linked to resistance to radiotherapy (34, 35). Low expression of MT in thyroid medullary carcinoma may reflect a distinct metabolic profile of thyroid C cells, from which the tumour originates.

The difference in MT expression between follicular adenoma and follicular carcinoma may indicate a potential to employ MT as an accessory marker, which would permit differentiation of the lesions. This would facilitate the difficult differential diagnosis of the two thyroid tumours, although, taking into account the numerous contradictory results related to expression of numerous MT isoforms in malignant thyroid lesions, further studies are needed.

## References

- 1 Jemal A, Thomas A, Murray T and Hun M: Cancer statistics, 2002. *CA Cancer J Clin* 52: 23-47, 2002.
- 2 Mazzaferri EL: Management of a solitary thyroid nodule. *N Engl J. Med* 328: 553-559, 1993.
- 3 Stojadinovic A, Ghossein RA, Hoos A, Urist MJ, Spiro RH, Shah JP, Brennan MF, Shaha AR and Singh B: Hürthle cell carcinoma: a critical histopathologic appraisal. *J Clin Oncol* 19: 2616-2625, 2001.
- 4 Dziągł P: Expression of metallothioneins in tumor cells. *Pol J Pathol* 55: 3-12, 2004.
- 5 Pedersen MØ, Larsen A, Stoltenberg M and Penkowa M: The role of metallothionein in oncogenesis and cancer prognosis. *Prog Histochem Cytochem* 44: 29-64, 2009.
- 6 Cherian MG and Apostolova MD: Nuclear localization of metallothionein during cell proliferation and differentiation. *Cell Mol Biol* 46: 347-356, 2000.
- 7 Koropatnick J, Kloth DM, Kadhim S, Chin JL and Cherian MG: Metallothionein expression and resistance to cisplatin in a human germ cell tumor cell line. *J Pharmacol Exp Ther* 275: 1681-1687, 1995.
- 8 Satoh M, Cherian MG, Imura N and Shimizu H: Modulation of resistance to anticancer drugs by inhibition of metallothionein synthesis. *Cancer Res* 54: 5255-5257, 1994.
- 9 Dziągł P, Suder E, Surowiak P, Kornafel J and Zabel M: Expression of metallothionein in synovial sarcoma cells. *Appl Immunohistochem Mol Morphol* 10: 357-363, 2002.
- 10 Surowiak P, Paluchowski P, Dziągł P, Wysocka T, Wojnar A, Spaczyński M and Zabel M: Lack of relationship between metallothionein expression and proliferation exponents of G2 grade of differentiation. *Med Sci Monit* 10: 300-305, 2004.

- 11 Dzięgiel P, Jeleń M, Muszyńska B, Maciejczyk A, Szulc A, Podhorska-Okołów M, Cegielski M and Zabel M: Role of Metallothionein expression in non-small cell lung carcinomas. *Rocz Akad Med Białymst* 49, Suppl 1: 43-45, 2005.
- 12 Szajerka A, Dzięgiel P, Szajerka T, Zabel M, Winowski J and Grzebieńiak Z: Immunohistochemical evaluation of metallothionein, MCM-2 and Ki-67 antigen expression in tumors of the adrenal cortex. *Anticancer Res* 28: 2959-2965, 2008.
- 13 Dzięgiel P, Salwa-Żurawska W, Żurawski J, Wojnar A and Zabel M: Prognostic significance of augmented metallothionein expression correlated with Ki-67 antigen expression in selected soft tissue sarcomas. *Histol Histopathol* 20: 83-89, 2005.
- 14 Rubio J, Ramos D, López-Guerrero JA, Iborra I, Collado A, Solsona E, Almenar S and Llombart-Bosch A: Immunohistochemical expression of Ki-67 antigen, Cox-2 and Bax/Bcl-2 in prostate cancer; prognostic value in biopsies and radical prostatectomy specimens. *Eur Urol* 48: 745-751, 2005.
- 15 Saad AG, Yeap BY, Thunnissen FB, Pinkus GS, Pinkus JL, Loda M, Sugarbaker DJ, Johnson BE and Chirieac LR: Immunohistochemical markers associated with brain metastases in patients with non small cell lung carcinoma. *Cancer* 113: 2129-2138, 2008.
- 16 Miglietta L, Vanella P, Canobbio L, Parodi MA, Guglielmini P and Boccardo F: Clinical and pathological response to primary chemotherapy in patients with locally advanced breast cancer grouped according to hormonal receptors, Her2 status, grading and Ki-67 proliferation index. *Anticancer Res* 29: 1621-1625, 2009.
- 17 Remmele W and Stegner HE: Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathologie* 8: 138-140, 1987.
- 18 Dzięgiel P, Dumańska M, Forgacz J, Wojnar A and Zabel M: Intensity of apoptosis as related to the expression of metallothionein (MT), caspase-3 (cas-3) and Ki-67 antigen and the survival time of patients with primary colorectal adenocarcinomas. *Rocz Akad Med Białymst* 49 Suppl 1: 5-7, 2004.
- 19 Dzięgiel P, Forgacz J, Suder E, Surowiak P, Kornafel J and Zabel M: Prognostic significance of metallothionein expression in correlation with Ki-67 expression in adenocarcinomas of large intestine. *Histol Histopathol* 18: 401-407, 2003.
- 20 Szelachowska J, Dzięgiel P, Jeleń-Krzeszewska J, Jeleń M, Tarkowski R, Spytkowska B, Matkowski R and Kornafel J: Correlation of metallothionein expression with clinical progression of cancer in the oral cavity. *Anticancer Res* 29: 589-595, 2009.
- 21 Athanassiadou P, Bantis A, Gonidi M, Athanassiades P, Ageloniou E, Grapsa D, Nikolopoulou P and Patsouris E: The expression of metallothioneins on imprint smears of prostate carcinoma: correlation with clinicopathologic parameters and tumor proliferative capacity. *Tumori* 93: 189-194, 2007.
- 22 Jin R, Chow VT, Tan PH, Dheen ST, Duan W and Bay BH: Metallothionein 2A expression is associated with cell proliferation in breast cancer. *Carcinogenesis* 23: 81-86, 2002.
- 23 Surowiak P, Kaplenko I, Spaczyński M and Zabel M: The expression of metallothionein (MT) and proliferation intensity in ovarian cancers treated with cisplatin and paclitaxel. *Folia Morphol (Warsz)* 62: 493-495, 2003.
- 24 Nartey N, Cherian G and Banerjee D: Immunohistochemical localization of metallothionein in human thyroid tumors. *Am J Pathol* 129: 177-182, 1987.
- 25 Schmid KW, Greeff M, Hittmair A, Totsch M, Ofner D, Dockhorn-Dworniczak B, Bocker W and Jasani B: Metallothionein expression in normal, hyperplastic, and neoplastic thyroid follicular and parafollicular C cells using monoclonal antimetallithionein antibody E9. *Endocr Pathol* 5: 114-122, 1994.
- 26 Ferrario C, Lavagni P, Gariboldi M, Miranda C, Losa M, Cleris L, Formelli F, Pilotti S, Pierotti MA and Greco A: Metallothionein 1G acts as an oncosuppressor in papillary thyroid carcinoma. *Lab Invest* 88: 474-481, 2008.
- 27 Huang Y, de la Chapelle A and Pellegata NS: Hypermethylation, but not LOH, is associated with the low expression of MT1G and CRABP1 in papillary thyroid carcinoma. *Int J Cancer* 104: 735-744, 2003.
- 28 Huang Y, Prasad M, Lemon WJ, Hampel H, Wright FA, Kornacker K, LiVolsi V, Frankel W, Kloos RT, Eng C, Pellegata NS and de la Chapelle A: Gene expression in papillary thyroid carcinoma reveals highly consistent profiles. *Proc Natl Acad Sci USA* 98: 15044-15049, 2001.
- 29 Finn SP, Smyth P, Cahill S, Streck C, O'Regan EM, Flavin R, Sherlock J, Howells D, Henfrey R, Cullen M, Toner M, Timon C, O'Leary JJ and Sheils OM: Expression microarray analysis of papillary thyroid carcinoma and benign thyroid tissue: emphasis on the follicular variant and potential markers of malignancy. *Virchows Arch* 450: 249-260, 2007.
- 30 Durak I, Bayram F, Kavutcu M, Canbolat O and Oztürk HS: Impaired enzymatic antioxidant defense mechanism in cancerous human thyroid tissues. *J Endocrinol Invest* 19: 312-315, 1996.
- 31 Mano T, Shinohara R, Iwase K, Kotake M, Hamada M, Uchimuro K, Hayakawa N, Hayashi R, Nakai A, Ishizuki Y and Nagasaka A: Changes in free radical scavengers and lipid peroxide in thyroid glands of various thyroid disorders. *Horm Metab Res* 29: 351-354, 1997.
- 32 Liu ZM, Hasselt CA, Song FZ, Vlantis AC, Cherian MG, Koropatnick J and Chen GG: Expression of functional metallothionein isoforms in papillary thyroid cancer. *Mol Cell Endocrinol* 302: 92-98, 2009.
- 33 Liu ZM, Chen GG, Shum CK, Vlantis AC, Cherian MG, Koropatnick J and van Hasselt CA: Induction of functional MT1 and MT2 isoforms by calcium in anaplastic thyroid carcinoma cells. *FEBS Lett* 581: 2465-2472, 2007.
- 34 Deng DX, Cai L, Chakrabarti S and Cherian MG: Increased radiation-induced apoptosis in mouse thymus in the absence of metallothionein. *Toxicology* 134: 39-49, 1999.
- 35 Cai L and Cherian MG: Zinc-metallothionein protects from DNA damage induced by radiation better than glutathione and copper- or cadmium-metallothioneins. *Toxicol Lett* 136: 193-198, 2003.

Received July 19, 2010

Revised November 8, 2010

Accepted November 10, 2010