

# Metallothionein 1G acts as an oncosuppressor in papillary thyroid carcinoma

Cristina Ferrario<sup>1,2</sup>, Paola Lavagni<sup>1</sup>, Manuela Gariboldi<sup>1,2</sup>, Claudia Miranda<sup>1</sup>, Marco Losa<sup>3</sup>, Loredana Cleris<sup>4</sup>, Franca Formelli<sup>4</sup>, Silvana Pilotti<sup>3</sup>, Marco A Pierotti<sup>1,2,5</sup> and Angela Greco<sup>1</sup>

The molecular pathogenesis of tumors arising from the thyroid follicular epithelial cells, including papillary (PTC) and follicular thyroid carcinoma (FTC), is only partially understood, and the role of tumor suppressor genes has not yet been assessed. The metallothionein (*MT*) gene family encodes a class of metal-binding proteins involved in several cellular processes, and their expression is often deregulated in human tumors. Recently, downregulation of *MT* gene expression in PTC has been reported, suggesting a possible oncosuppressor role of this gene family in the pathogenesis of thyroid tumors. To further explore this possibility, we performed expression and functional studies. Analysis of microarray data of thyroid tumors of different histologic types showed that several *MT* genes were downregulated with respect to normal tissue. The microarray data were corroborated by quantitative PCR experiments, showing downregulation of *MTs* in PTC and FTC, but to a greater extent in papillary carcinoma. The expression of *MTs* was also investigated at the protein level by immunohistochemistry; the results were consistent with the microarray data, showing general downregulation in tumor samples, which was more evident in PTC. The functional consequence of *MT* downregulation was addressed employing an experimental model made of the PTC-derived K1 cell line in which *MT1G* expression is repressed by promoter methylation. Restoration of *MT1G* expression by cDNA transfection affected growth rate and *in vivo* tumorigenicity of K1 cells, indicating an oncosuppressor role for *MT1G* in thyroid papillary tumorigenesis.

*Laboratory Investigation* (2008) 88, 474–481; doi:10.1038/labinvest.2008.17; published online 10 March 2008

**KEYWORDS:** gene expression; K1 cell line; metallothionein; papillary thyroid carcinoma; thyroid carcinogenesis

Several tumor types, differing in biological and clinical behavior, originate from the thyroid epithelial follicular cells. They include well-differentiated, indolent papillary thyroid and follicular thyroid carcinomas (PTC and FTC), as well as extremely aggressive anaplastic carcinoma.<sup>1</sup> Studies performed in several laboratories, including ours, have demonstrated that distinct molecular events are associated with specific tumor types.<sup>2</sup> FTC is characterized by the *PAX8/PPAR $\gamma$*  rearrangement and activating mutations of *RAS* genes.<sup>1</sup> PTC is associated with rearrangements involving the *RET* and *NTRK1* tyrosine kinase receptors,<sup>2</sup> and the V600E *BRAF*-activating mutation.<sup>3–5</sup> More recently, microarray studies have identified several genes that might be important in the molecular pathogenesis and the malignant progression of thyroid cancer and could be used as diagnostic or prognostic molecular markers. These candidate genes are involved

in several different processes, such as cell adhesion, cell cycle progression, mitogenic control and tumorigenesis.<sup>6–10</sup> In spite of that, the molecular pathogenesis of thyroid cancer is still incomplete; in particular, a role of tumor suppressor genes has not yet been assessed. Metallothioneins (*MTs*) are low-molecular weight proteins of 6–7 kDa, with high content of cysteine (30%) and complete absence of aromatic amino acids and histidine<sup>11</sup> capable of binding heavy metals with high affinity. Human *MTs* are encoded by a family of genes clustered on chromosome 16 and containing at least 10 functional members, and are organized in four major isoforms: *MT1* (seven functional genes), *MT2*, *MT3* and *MT4*.<sup>12</sup> *MT1* and *MT2* are ubiquitously expressed, and *MT2* accounts for 80% of *MT* expression, at least in some tissues.<sup>13</sup> Expression of *MT3* and *MT4* is restricted to brain and reproductive organs, and to stratified squamous epithelia of

<sup>1</sup>Operative Unit 'Molecular Mechanisms of Cancer Growth and Progression', Department of Experimental Oncology, Fondazione IRCCS 'Istituto Nazionale dei Tumori', Milan, Italy; <sup>2</sup>IFOM Foundation (The Firc Institute for Molecular Oncology Foundation), Milan, Italy; <sup>3</sup>Department of Pathology, Experimental Molecular Pathology, Fondazione IRCCS 'Istituto Nazionale dei Tumori', Milan, Italy; <sup>4</sup>Operative Unit 'Chemoprevention', Department of Experimental Oncology, Fondazione IRCCS 'Istituto Nazionale dei Tumori', Milan, Italy and <sup>5</sup>Scientific Direction, Fondazione IRCCS 'Istituto Nazionale dei Tumori', Milan, Italy  
Correspondence: Dr A Greco, PhD, Operative Unit 'Molecular Mechanisms of Cancer Growth and Progression', Department of Experimental Oncology Fondazione IRCCS 'Istituto Nazionale dei Tumori', Via G Venezian, 1, Milan 20133, Italy. E-mail: angela.greco@istitutotumori.mi.it

Received 06 November 2007; revised 25 January 2008; accepted 29 January 2008

skin and tongue, respectively. While *MT3* and *MT4* are constitutively expressed, *MT1* and *MT2* are coordinately regulated by a variety of developmental and environmental signals, such as metals, oxidative stress, cytokines and glucocorticoid hormones.<sup>14</sup> The primary functions of MTs have not been defined with certainty. They regulate the availability of heavy metals, especially zinc, which associates with hundreds of proteins regulating gene expression, metabolism and cell growth. MTs also participate in metal detoxification and protection against oxidative stress.<sup>12,14</sup>

The role and the expression of MTs in human tumors have been extensively investigated, but the results are often contradictory among different cancer types; therefore, the real contribution of these important molecules to malignant transformation has not yet been clarified.<sup>15</sup> *MT* overexpression has been observed in several tumors such as melanoma, breast and lung cancer, and in some cases has been associated with a poor prognosis<sup>16,17</sup> and with resistance to treatments.<sup>18</sup> Conversely, other tumor types, such as colorectal cancer and hepatocellular carcinoma, show a downregulation of *MT* expression during cancer progression.<sup>19,20</sup> Recently, the molecular mechanism for the downregulation of *MT* genes in liver tumors has been unveiled; it involves the PI3K cascade.<sup>21</sup> With respect to thyroid tumors, by microarray analysis Huang *et al*<sup>6</sup> identified *MTs* as genes downregulated in PTC with respect to normal thyroid tissue. Subsequently, they showed that in PTC the expression of *MT1G* gene is repressed by promoter methylation, suggesting an oncosuppressor role.<sup>22</sup> In agreement with this finding, immunohistochemistry analysis showed reduced *MT* expression in PTC.<sup>23</sup> To further define the role of *MTs* in thyroid carcinogenesis, we performed expression and biological studies. The expression of several *MT* genes in a collection of normal and tumor tissue samples was investigated by analyzing gene expression profile data and by performing quantitative real-time PCR (QRT-PCR). In addition, by immunohistochemistry, *MT* expression was also investigated at the protein level. Both approaches documented downregulation of several *MT* genes in PTC and, to a lesser extent, in FTC. The functional consequences of *MT* downregulation were addressed in K1 cells, in which the expression of *MT1G* is silenced by promoter methylation. Restoration of *MT1G* expression by cDNA transfection reduced *in vitro* growth rate and tumorigenicity of K1 cells. Overall, our data suggest that *MT* genes act as oncosuppressors in thyroid epithelial carcinogenesis.

## MATERIALS AND METHODS

### Sample Collection and Microarray Analysis

The thyroid samples were collected at the Department of Pathology at the 'Istituto Nazionale dei Tumori' (Milan, Italy) from 1984 to date. All the patients gave their written informed consent. The specimens were snap frozen. A frozen section of each cryopreserved sample was stained with hematoxylin and eosin, and samples eligible for microarray

analysis, ie containing exclusively tumor or normal tissue, were selected. The cDNA microarray used in this study contains 4451 unique clones selected from the human sequence-verified IMAGE clone collection (Research Genetics/Invitrogen, Carlsbad, CA, USA) along with plant genes, printing controls (water/DMSO) and spike genes (Amersham Bioscience, Amersham, UK) added as internal controls. Each cDNA fragment was PCR-amplified and spotted in triplicate on type 7 star slides (Amersham Bioscience, UK). The procedures of RNA isolation, probe labeling, sample hybridization and scanning were performed as previously described.<sup>24</sup> Supervised analysis (class comparison and hierarchical clustering) was performed using BRB ArrayTool v.3.3\_Beta1 (<http://linus.nci.nih.gov/BRB-ArrayTools.html>).

The expression levels of the three *MT* genes in all the tissue samples hybridized on the array were measured as log ratio between the expression level of the specimens and that of the reference. Genes showing a positive log ratio value (ie more expressed in the thyroid sample than in the reference) are called upregulated; those with a negative log ratio value are called downregulated.

### QRT-PCR

Total RNA from thyroid tissues was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Primers specific for the different *MT* isoforms, located in the divergent 5' and 3' untranslated regions, were used.<sup>25</sup> PCR products were subjected to direct nucleotide sequencing, and their correct identity was confirmed using BLAST software (<http://www.ncbi.nlm.nih.gov/blast>). The housekeeping gene *18S* was amplified using the following primers: Fw 5'-CGCCGCTAGAGGTGAAATTC-3', Rev 5'-CTTTCGCTCTGGTCCGTCTT-3'.<sup>26</sup> QRT-PCR reactions were carried out in triplicate using 2 × SYBR GREEN PCR Master Mix (Applied Biosystems) on the ABI PRISM 7700 Sequence Detector System (Applied Biosystems) and the amplification conditions reported by Tai *et al*.<sup>27</sup> Relative quantification of expression levels was calculated using the comparative  $C_t$  method;<sup>28</sup> for each *MT* isoform, the normal sample showing the lowest expression was selected as a calibrator for the calculation of the  $\Delta\Delta C_t$  value; samples with an expression level lower than the calibrator were called downregulated.

### Immunohistochemistry

Formalin-fixed and paraffin-embedded 2- $\mu$ m tumor sections were treated with 3% H<sub>2</sub>O<sub>2</sub> to inhibit endogenous peroxidase, and then subjected to antigen retrieval using 5 mM citrate buffer (pH 6) in autoclave at 95°C for 15 min. Sections were incubated with Ultra V Block (Lab Vision, Fremont, CA, USA) for 10 min, to limit nonspecific reactivity, and then incubated with *MT* mouse monoclonal antibody (clone E9; Dako, Carpinteria, CA, USA) diluted 1:150 in ChemMate™ antibody diluent (Dako) for 1 h. All stains were carried out by primary antibody enhancer and polymer following provided

protocol (UltraVision LP large-volume detection system HRP polymer; Lab Vision) and developed with liquid DAB + substrate chromogen system (Dako). Breast was used as positive control. A semiquantitative assessment of the MT immunostaining was determined using the multiplicative 'quick-score' method, which accounts for both the intensity and the extent of cell staining.<sup>29</sup> To obtain the relative expression, the score values of each sample were normalized for that of normal tissue.

### Constructs

The *MT1G* cDNA was isolated by RT-PCR using the primers described by Tai *et al*<sup>27</sup> and cloned into the pTarget expression vector. The insert was extracted by *XhoI/EcoRI* digestion and transferred into the *XhoI/EcoRI*-cut pcDNA3 expression vector. The resulting construct carries the *MT1G* cDNA in the sense orientation with respect to the CMV promoter present in the vector. This plasmid was used as a template for the production of a myc-tagged cDNA. The *MT1G* cDNA was amplified with primers T7 (AATACGACTCACTATAGGG) and **GCGGATCCGGCGCAGCAGCTGCACTTCTC**, containing nucleotides 166–183 of *MT1G* mRNA (italic), and the *Bam*HI restriction site (bold). The PCR product was digested with *Eco*RI and *Bam*HI endonucleases and inserted into the pcDNA3.1/myc-His(-)A carrying compatible ends. The resulting construct encodes the MT1G-myc fusion protein.

### Cell Culture and Transfection

The K1 cell line, derived from a human PTC and obtained from ECACC, was cultured at 37°C, 5% CO<sub>2</sub> humidified atmosphere, in DMEM:HAM'S F12:MCDB 105 (2:1:1) containing 2 mM glutamine and 10% FCS. Transient transfection was performed with Fugene6 (Roche, Indianapolis, IN, USA) following manufacturer's instructions. Stable transfection was performed with Nucleofector™ II (Amaxa Biosystem, Gathesburg, MD, USA). Plasmid DNA (1 µg) (pcDNA3.1-MT1G-myc or pcDNA3.1-myc, as control) was added to a mix containing 90 µl of human keratinocyte nucleofector solution and 20 µl of Supplement 1 Human Keratinocyte Nucleofector Kit (Amaxa Biosystem). K1 cells (1 × 10<sup>6</sup>) were resuspended in the above solution and were transfected by applying the T003 program using the Nucleofector™ II (Amaxa Biosystem). Transfected cells were seeded onto two 10 cm Petri dishes, and G418 selection (400 µg/ml) was applied 24 h later. G418-resistant colonies were isolated after 2 weeks of selection and propagated in G418-containing medium.

### Growth Curves

To determine the growth rate of K1-MT1G-myc clones, the alamarBlue® Assay (Biosource) was used, following the supplier information. Fifty cells per well were plated onto 96-well multiwell (Corning). After adhesion of cells, the alamarBlue (Biosource) was added to the medium at 20% v/v. The fluorescence at λ = 590 and 535 nm was detected

every 24 h, from day 1 to day 8, using a microplate reader (Tecan).

### Western Blot Analysis

Proteins were extracted in RIPA buffer, quantified by Bradford's assay with the BIO-RAD Protein Assay (Bio-Rad, Munchen, Germany). Total protein extracts (70–100 µg) were separated on NuPAGE 4–12% Bis-Tris precasted gel (Invitrogen) and blotted on Hybond™-C supermembrane (Amersham Bioscience, Uppsala, Sweden). Hybridization was carried out with anti-MT E9 antibody (Dako, Carpinteira, CA, USA) or with the anti-myc 9E10 antibody hybridoma (Roche) and with the anti-β actin antibody (Sigma, St Louis, MO, USA). The immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Bioscience, Sweden).

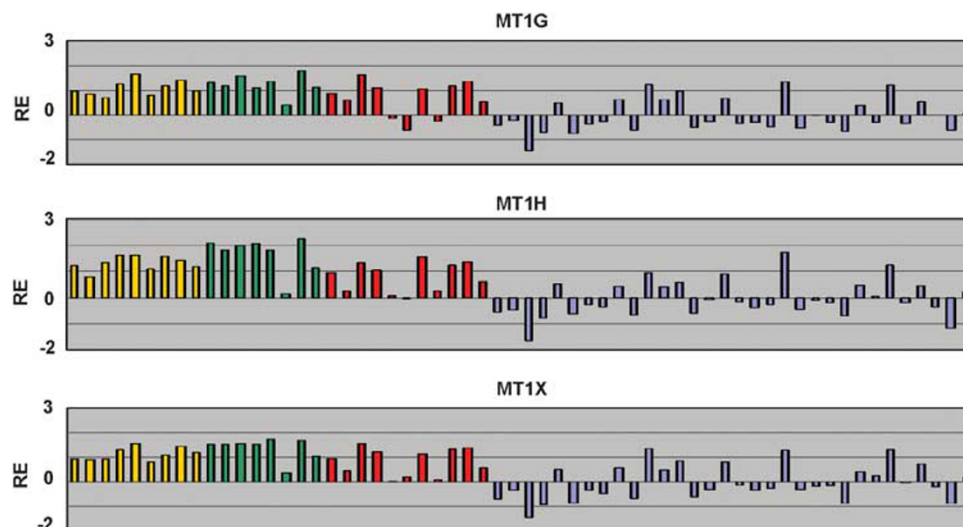
### In Vivo Studies

Animal studies were reviewed and approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS 'Istituto Nazionale dei Tumori' and are in accordance with the guidelines of the UK Coordinating Committee for Cancer Research.<sup>30</sup> Female CD-1 nu/nu mice (8- to 9-week old) (Charles River, Calco, Italy) were injected subcutaneously into the left flank with 5 × 10<sup>6</sup> cells in 0.2 ml PBS volume. Tumor growth was assessed by evaluating tumor latency, ie days to reach 0.1 g, and by monitoring tumor weight (TW) twice a week. TW was estimated by the formula  $TW(g) = d^2 \times D/2$  where *d* and *D* are the shortest and the longest diameters of the tumor, respectively, measured in cm.

## RESULTS

### Analysis of MT Gene Expression in Thyroid Tumors

We explored the expression level of *MT* genes by analyzing cDNA microarray data produced in our laboratory using RNA from a collection of 59 thyroid samples, including 9 normal thyroid tissues (N), 8 follicular adenomas (FA), 11 FTCs and 31 PTCs. RNAs were hybridized to a microarray spotted with 4451 unique cDNA clones; a pool of 10 human cell lines was used as a reference.<sup>24</sup> Gene expression levels of the tumor histotype were compared to that of normal thyroid using class comparison analysis (<http://linus.nci.nih.gov/BRB-ArrayTools.html>). The *MT1G*, *MT1H* and *MT1X* genes were identified within a group of genes with low expression levels and strong downregulation in PTC samples compared to normal tissue (data not shown). Analysis of the expression levels of the three *MT* genes in all the tissue samples hybridized on the array are reported in Figure 1 and showed that the different *MT* isoforms are downregulated in at least 19 out of 31 (60%) of PTC and 3 out of 11 (27%) of FTC. Our findings confirm the previous data on *MT* RNA downregulation in PTC<sup>6</sup> and, in addition, they also suggest that MTs may be downregulated in FTC.



**Figure 1** cDNA array expression of *MT* genes. Graphic representation of the expression levels of the three *MT* clones spotted on the cDNA array in normal thyroid samples (yellow), follicular adenomas (green), follicular carcinomas (red) and papillary carcinomas (blue). The expression levels (relative expression, RE) of the three *MT* genes in all the tissue samples hybridized on the array were measured as log ratio between the expression level of the specimens and that of the reference. Genes showing a positive log ratio value (ie more expressed in the thyroid sample than in the reference) are called upregulated; those with a negative log ratio value are called downregulated.

To corroborate the microarray data, we performed a QRT-PCR experiment with primers located on the divergent 5' and 3' untranslated regions of each *MT* gene; these primers do not cross react with other members of the family and thus are able to discriminate among the different *MT* isoforms.<sup>25</sup> In addition to the three *MT1* genes present on the array, we also selected primers for the analysis of the expression of *MT1A*, *MT1B*, *MT1E*, *MT1F* and *MT2A*, thus including all the *MT* genes ubiquitously expressed in human tissues. Informative results (based on expression level and/or reproducibility) were obtained for *MT1E*, *MT1G*, *MT1X* and *MT2A*. The  $\Delta\Delta C_t$  calculation was used to determine the expression level of the samples analyzed by QRT-PCR; the normal sample showing the lowest expression level was used as a calibrator. Samples with an expression level lower than the calibrator were called downregulated, and their frequencies are reported in Table 1. *MT1E* is downregulated in 80.9% of PTC and in 50% of FTC; *MT1G* is downregulated in 60% of PTC and 9% of FTC; *MT1X* is downregulated only in PTC (76%); *MT2A* shows downregulation in PTC (65%) and FTC (40%). On the whole these data indicate that (a) *MT* downregulation occurs frequently in differentiated epithelial thyroid tumors, being detectable in 38–71% of the cases analyzed, (b) although involving both differentiated follicular thyroid cancer types, *MT* downregulation is prevalent in PTC and (c) *MT* downregulation is infrequent in FA, since only a single specimen showing *MT2A* downregulation was detected (data not shown).

Our results are in keeping with a previous observation reporting *MT* downregulation in PTC<sup>6</sup> and, interestingly, they provide also important information about other *MT* isoforms and other thyroid tumor histotypes.

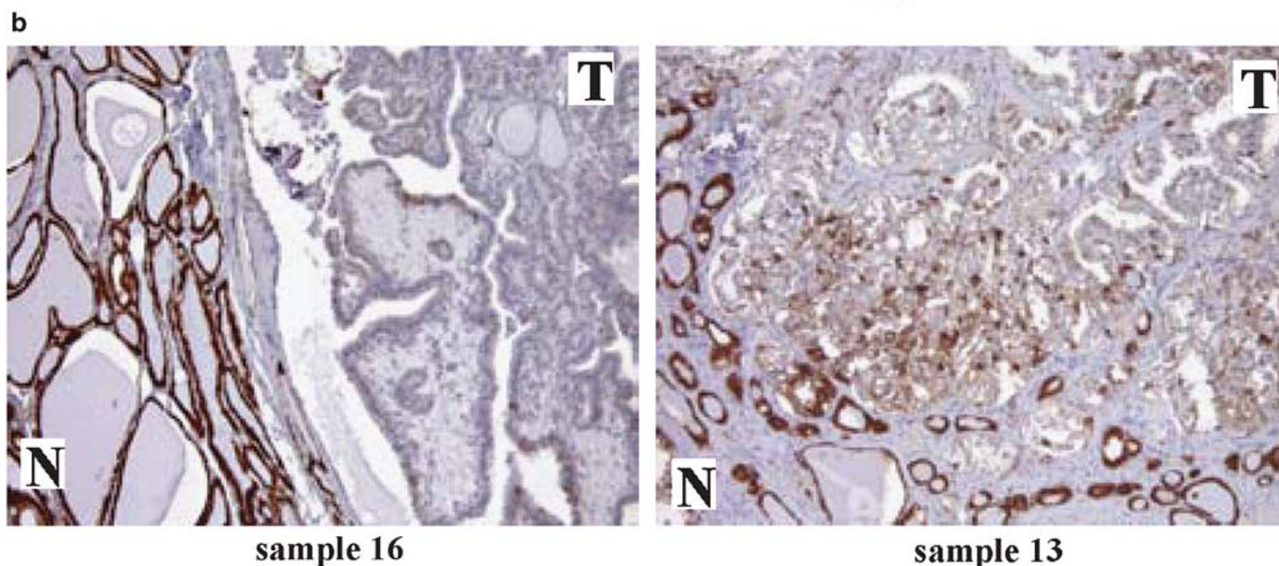
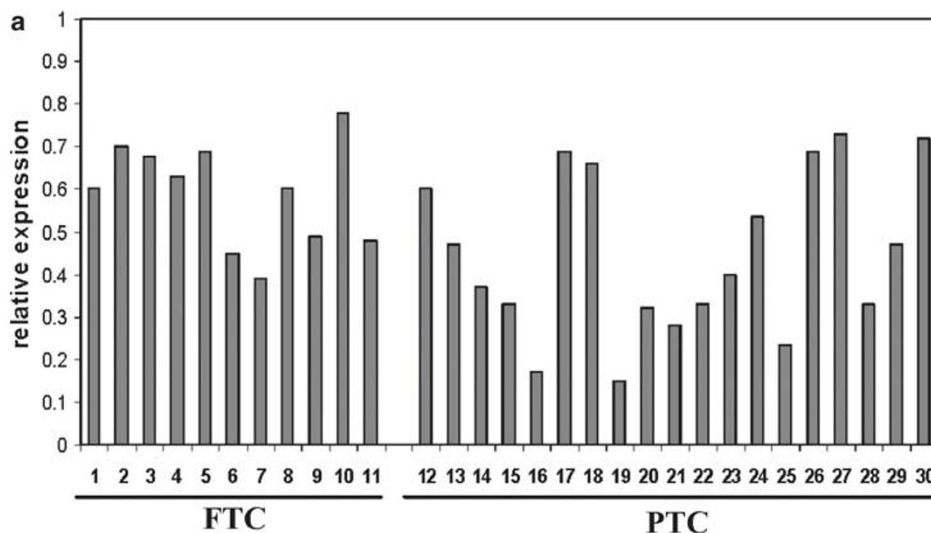
**Table 1** *MT* downregulation in differentiated thyroid tumors

	PTC (%)	FTC (%)	Total (%)
<i>MT1E</i>	17/21 (80.9)	5/10 (50)	22/31 (71)
<i>MT1G</i>	9/15 (60)	1/11 (9)	10/26 (38.4)
<i>MT1X</i>	16/21 (76)	0/10	16/31 (51.6)
<i>MT2A</i>	13/20 (65)	4/10 (40)	17/30 (57)

Frequency of downregulation of different *MT* isoforms in PTC and FTC with respect to normal thyroid tissue. After QRT-PCR,  $\Delta\Delta C_t$  calculation was used to determine the expression level of the samples analyzed and samples with an expression level lower than the calibrator (the normal sample showing the lowest expression level) were called downregulated.

### Evaluation of *MT* Protein Expression by Immunohistochemistry

With respect to expression analysis at protein level, two contradictory studies show either overexpression or downregulation of *MT* proteins in PTC as compared to normal thyroid.<sup>31</sup> We investigated the *MT* protein expression by immunohistochemistry in the same samples analyzed by microarray. Corresponding archival paraffin-embedded sections, including both tumor and normal tissue were selected, and immunohistochemistry was performed using the E9 mouse monoclonal antibody, which reacts with both *MT1* and *MT2* isoforms. Normal thyroid follicular cells showed an intense staining, whereas in the tumor counterpart a heterogeneous staining pattern was observed. A semiquantitative assessment of the *MT* immunostaining was determined using the multiplicative 'quick-score' method,<sup>29</sup> and the *MT* expression level of each sample with respect to



**Figure 2** Detection of MT expression by immunohistochemistry. (a) Immunohistochemistry was performed on 2- $\mu$ m cut formalin-fixed and paraffin-embedded sections as described in Materials and methods. A semiquantitative assessment of the MT immunostaining was determined using the multiplicative ‘quick-score’ method, which accounts for both the intensity and the extent of cell staining. The relative expression was obtained by normalizing the score values of each sample for that of normal tissue. (b) Section of two PTC samples (13 and 16) with different extent of MT downregulation. The normal (N) and tumor (T) components are indicated.

normal thyroid cells was calculated. The results are shown in Figure 2a. All the tumor samples showed reduced MT expression compared to normal controls. In the FTC series, the reduction was 22–61%; in one sample only, it was greater than 60%. In the PTC series, reduction was in the range 28–75%, and it was greater than 60% in 9 out 19 samples. In Figure 2b, two PTC samples with different extent (53 and 83%, respectively) of MT protein downregulation are shown. On the whole, the immunohistochemistry analysis demonstrates that the overall expression of MT proteins is frequently decreased in thyroid tumors, particularly in PTC. Our results are in agreement with a recent report<sup>22</sup> in which the same antibody was used, and demonstrate that *MT* gene expression downregulation correlate with reduced protein level.

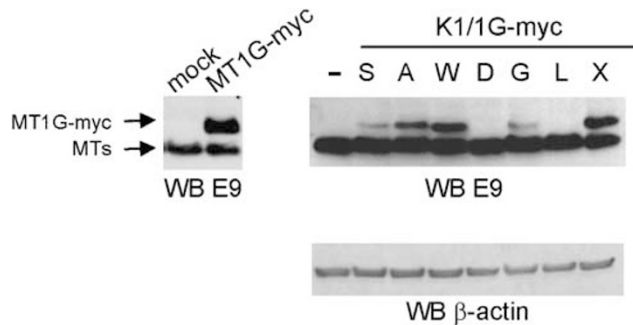
**Restoration of *MT1G* Gene Expression in K1 Thyroid Carcinoma Cell Line**

The K1 cell line, derived from a PTC, does not express the *MT1G* gene as a consequence of promoter methylation, as demonstrated by its re-expression following azacytidine treatment<sup>22</sup> (our unpublished results). Other *MT* isoforms are expressed, as detected by western blot analysis with E9 antibody (Figure 3). To assess the role of *MT* downregulation in PTC, we restored the expression of *MT1G* in K1 cells. We constructed the *MT1G*-myc expression plasmid carrying the *MT1G* cDNA fused in frame with the myc epitope and producing a 9 kDa *MT1G*-myc protein detected by both anti-myc and anti-MT (E9) antibodies and, in the latter case, distinguishable from the endogenous MT proteins (6 kDa)

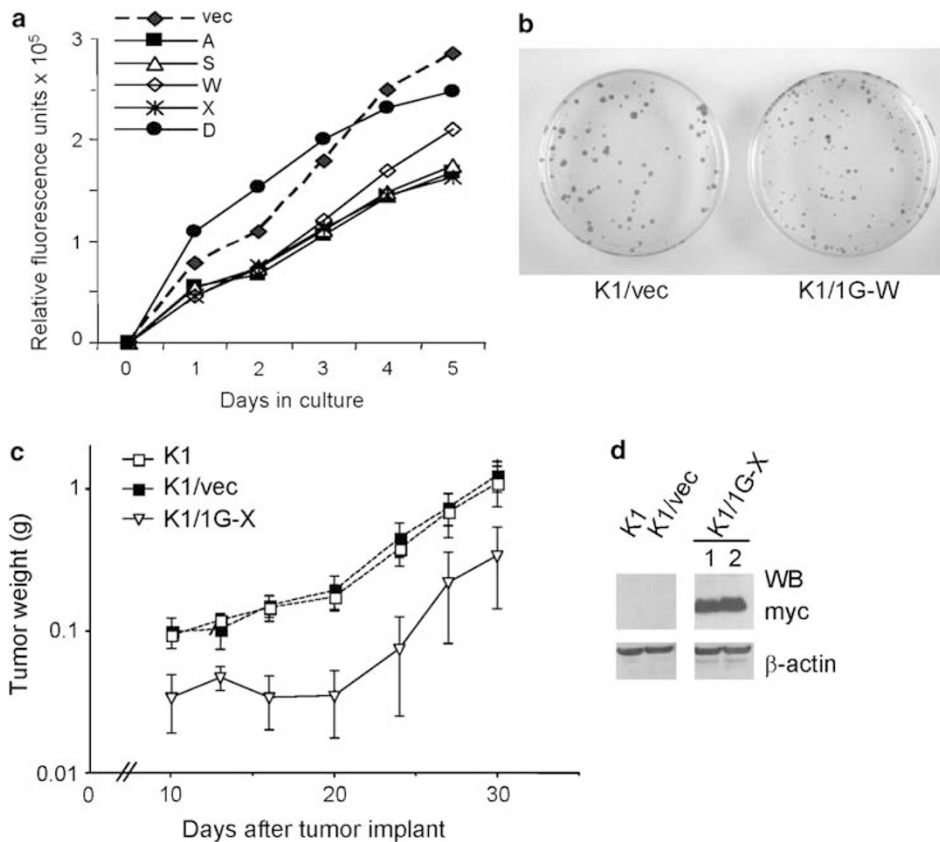
(Figure 3). The MT1G-myc expression plasmid was transfected into K1 cells, and transfected clones were isolated by selection in geneticin. Western blot analysis identified several independent clones expressing the exogenous MT1G-myc

protein at variable levels (Figure 3, right). To investigate the effect of *MT1G* restoration in K1 cells, the growth rate of K1/MT1G-myc clones (namely K1/1G-A, -S, -W and -X) was determined. As a control, the K1/1G-D clone (not expressing MT1G-myc) and the K1/vec clone (transfected with the empty vector) were used. As shown in Figure 4a, clones expressing the MT1G-myc protein display a growth rate reduced with respect to control cells. As a consequence, when plated at low density K1/1G cells formed colonies smaller than those of control cells; an example is shown in Figure 4b.

We next investigated the impact of MT1G-induced growth reduction on the *in vivo* tumorigenicity of K1 cells by inoculating K1/1G-X cells in nude mice. As a control, both the parental K1 cell line and the K1/vec clone were used. For each cell line,  $5 \times 10^6$  cells were injected subcutaneously. TW was monitored from day 10 to day 30 after implant; the results are reported in Figure 4c. The control cells induced tumor formation in 4/4 mice with tumor latency of 10 days, and none of the tumors regressed. The K1/1G-X cell line induced tumor formation in 4/5 mice; among the tumor-bearing mice, one underwent complete remission. The growth rate of



**Figure 3** Expression of MT1G-myc protein in K1 cells. K1 cells transiently transfected with MT1G-myc construct (left) and K1 clones stably transfected with MT1G-myc construct (right) were analyzed by western blot for the expression of MT1G-myc protein using the E9 anti-MT antibody. As a control, untransfected (-) or mock-transfected K1 cells were used. Endogenous MTs and the MT1G-myc proteins are indicated by arrows.



**Figure 4** Effect of MT1G-myc protein expression in K1 cells. (a) Growth curve of K1/1G and K1/vec clones were determined by the alamarBlue method as described in the Materials and methods section. Fluorescence was determined at the indicated time points. (b) Colonies formed by the K1/1G-W and by the K1/vec control clone are shown. In total, 600 cells were plated onto 10 cm Petri dishes; colonies were fixed and stained after 2 weeks. (c) *In vivo* tumorigenicity assay. K1/1G-X, K1/vec clones and parental K1 cells were inoculated subcutaneously into the left flank of athymic nude mice as described in Materials and methods. Tumor growth was monitored twice a week from day 10 to day 30. (d) The expression of MT1G-myc protein in K1/1G-X cells, before (1) and after (2) implant in mice was determined by western blot with anti-myc antibodies. As a control, parental K1 and K1/vec cells are shown.



tumors induced by K1/IG-X cells was reduced with respect to that of tumors induced by parental K1 and K1/vec cell lines. At day 30 mice were killed; tumors were explanted and used for cell culture establishment. Western blot analysis showed that tumor-derived cells express the MT1G-myc protein (Figure 4d). These results demonstrate that restoration of *MT1G* expression reduces the tumorigenicity of K1 thyroid carcinoma cells.

## DISCUSSION

By expression and functional studies, we have provided evidence that *MT* genes may act as tumor suppressor in thyroid tumorigenesis.

Expression data (array and QRT-PCR) showed that downregulation of *MT* gene expression (namely *MT1E*, *MT1G*, *MT1X* and *MT2A*) is associated with both thyroid differentiated tumor types, although it appears prevalent in PTC. Our results are in keeping with a previous observation reporting *MT* downregulation in PTC<sup>6</sup> and, interestingly, they also provide important information about other *MT* isoforms and other thyroid tumor histotypes. Indeed, they demonstrate that expression of *MT* isoforms can be modulated to different extents in different thyroid tumor histotypes. While this paper was in preparation, a paper reported downregulation of 13 different *MT* genes in PTC,<sup>32</sup> in agreement with our study and the previous report by Huang *et al.*<sup>6</sup>

At variance with the other two studies, we also investigated the *MT* expression at the protein level, by immunohistochemistry. In this respect, two contradictory previous studies are available. An early report<sup>31</sup> showed that *MTs* are higher expressed in PTC than in normal thyroid tissues, whereas a more recent study documented the absence of *MT* expression in a consistent fraction (65%) of PTC.<sup>23</sup> Our data are in keeping with the second report: we detected a considerable reduction of *MT* expression in PTC, in agreement also with gene expression studies. The discrepancy with the early study is most likely related to the different antibodies used.

The mechanism(s) leading to *MT* mRNA downregulation in tumors may involve *MT* promoter methylation as well as transcriptional repression. With respect to PTC, Huang *et al.*<sup>22</sup> have shown that repression of *MT1G* is associated with promoter downregulation.

The molecular consequences of *MT* downregulation in some cancers, including thyroid carcinoma, are not completely understood. Considering the antioxidant role of *MTs*, their downregulation should have a detrimental effect for the cell. However, this could be overcome by the activation of compensatory mechanisms. On the other hand, the lack of *MTs* could result in greater availability of zinc for a variety of zinc-finger transcription factor, whose increased activity could account for the rapid proliferation of cancer cells. Moreover, *MT* downregulation may act together with

deregulation of other genetic elements in promoting tumor cell growth.

To explore the contribution of *MT* downregulation to thyroid tumor phenotype, we restored the *MT1G* gene in the K1 cell line, in which the endogenous gene is silenced by promoter methylation,<sup>22</sup> whereas other *MT* isoforms are expressed. With respect to the parental K1 cells, K1 clones expressing exogenous *MT1G* showed reduction of both *in vitro* proliferation rate and *in vivo* tumorigenicity, thus demonstrating that *MT1G* counteracts the growth properties of transformed cells. It is worth noting that K1 cells harbor the V600E BRAF mutation, which has been recently shown to be important to maintain proliferation, transformation and tumorigenicity of BRAF-mutant PTC cell lines.<sup>33</sup> Our data demonstrate that even in the presence of the V600E BRAF mutation and of expression of several other *MT* isoforms, restoration of *MT1G* expression is capable of modulating the transformed properties of K1 cells.

Taken together, our results demonstrate that low *MT* gene expression is associated with PTC, and that *MTs* could play an oncosuppressor role in thyroid carcinogenesis. However, further studies are needed to elucidate the precise contribution of *MTs* to thyroid carcinogenesis. These involve the dissection of the role of each *MT* isoform, and the determination of possible cross-talk between *MT* downregulation and intracellular pathways triggered by the various PTC-associated oncogenes.

## ACKNOWLEDGEMENT

We thank Dr Loris De Cecco, Dr James Reid and Dr Lara Lusa for their contribution to microarray analysis; we also thank Sonia Pagliardini for technical assistance. This study was financially supported by AIRC (Italian Association for Cancer Research) and Ministry of Health RF2004 Grant no. 90.

1. Sherman SI. Thyroid carcinoma. *Lancet* 2003;361:501–511.
2. Pierotti MA, Bongarzone I, Borrello MG, *et al.* Cytogenetics and molecular genetics of the carcinomas arising from the thyroid epithelial follicular cells. *Genes Chromosome Cancer* 1996;16:1–14.
3. Xu X, Quiros RM, Gattuso P, *et al.* High prevalence of BRAF gene mutation in papillary thyroid carcinomas and thyroid tumor cell lines. *Cancer Res* 2003;63:4561–4567.
4. Kimura ET, Nikiforova MN, Zhu Z, *et al.* High prevalence of BRAF mutations in thyroid cancer: gene evidence for constitutive activation of RET/PTC–RAS–BRAF signaling pathway in papillary thyroid carcinoma. *Cancer Res* 2003;63:1454–1457.
5. Frattini M, Ferrario C, Bressan P, *et al.* Alternative mutations of *BRAF*, *RET* and *NTRK1* are associated with similar but distinct gene expression patterns in papillary thyroid cancer. *Oncogene* 2004;23:7436–7440.
6. Huang Y, Prasad M, Lemon WJ, *et al.* Gene expression in papillary thyroid carcinoma reveals highly consistent profiles. *Proc Natl Acad Sci USA* 2001;98:15044–15049.
7. Barden CB, Shister KW, Zhu B, *et al.* Classification of follicular thyroid tumors by molecular signature: results of gene profiling. *Clin Cancer Res* 2003;9:1792–1800.
8. Aldred MA, Huang Y, Liyanarachchi S, *et al.* Papillary and follicular thyroid carcinomas show distinctly different microarray expression profiles and can be distinguished by a minimum of five genes. *J Clin Oncol* 2004;22:3531–3539.
9. Jarzab B, Wiench M, Fijarewicz K, *et al.* Gene expression profile of papillary thyroid cancer: sources of variability and diagnostic implications. *Cancer Res* 2005;65:1587–1597.

10. Giordano TJ, Kuick R, Thomas DG, *et al*. Molecular classification of papillary thyroid carcinoma: distinct BRAF, RAS, and RET/PTC mutation-specific gene expression profiles discovered by DNA microarray analysis. *Oncogene* 2005;24:6646–6656.
11. Fischer EH, Davie EW. Recent excitement regarding metallothionein. *Proc Natl Acad Sci USA* 1998;95:3333–3334.
12. Cherian MG, Jayasurya A, Bay BH. Metallothioneins in human tumors and potential roles in carcinogenesis. *Mutat Res* 2003;533:201–209.
13. Hunziker PE. Purification of human isometallothioneins. *Methods Enzymol* 1991;205:244–247.
14. Haq F, Mahoney M, Koropatnick J. Signaling events for metallothionein induction. *Mutat Res* 2003;533:211–226.
15. Theocharis SE, Margeli AP, Klijanienko JT, *et al*. Metallothionein expression in human neoplasia. *Histopathology* 2004;45:103–118.
16. Weinlich G, Bitterlich W, Mayr V, *et al*. Metallothionein-overexpression as a prognostic factor for progression and survival in melanoma. A prospective study on 520 patients. *Br J Dermatol* 2003;149:535–541.
17. Joseph MG, Banerjee D, Kocha W, *et al*. Metallothionein expression in patients with small cell carcinoma of the lung: correlation with other molecular markers and clinical outcome. *Cancer* 2001;92:836–842.
18. Chun JH, Kim HK, Kim E, *et al*. Increased expression of metallothionein is associated with irinotecan resistance in gastric cancer. *Cancer Res* 2004;64:4703–4706.
19. Deng DX, Chakrabarti S, Waalkes MP, *et al*. Metallothionein and apoptosis in primary human hepatocellular carcinoma and metastatic adenocarcinoma. *Histopathology* 1998;32:340–347.
20. Stenram U, Ohlsson B, Tranberg KG. Immunohistochemical expression of metallothionein in resected hepatic primary tumors and colorectal carcinoma metastases. *APMIS* 1999;107:420–424.
21. Datta J, Majumder S, Kutay H, *et al*. Metallothionein expression is suppressed in primary human hepatocellular carcinomas and is mediated through inactivation of CCAAT/enhancer binding protein alpha by phosphatidylinositol 3-kinase signaling cascade. *Cancer Res* 2007;67:2736–2746.
22. Huang Y, de la Chapelle A, Pellegata NS. Hypermethylation, but not LOH, is associated with the low expression of MT1G and CRABP1 in papillary thyroid carcinoma. *Int J Cancer* 2003;104:735–744.
23. Schmid KW, Greeff M, Hittmair A, *et al*. Metallothionein expression in normal, hyperplastic, and neoplastic thyroid follicular and parafollicular C cells using monoclonal antimetallothionein antibody E9. *Endocrinol Pathol* 1994;5:114–122.
24. De Cecco L, Marchionni L, Gariboldi M, *et al*. Gene expression profiling of advanced ovarian cancer: characterization of a molecular signature involving fibroblast growth factor 2. *Oncogene* 2004;23:8171–8183.
25. Mididoddi S, McGuiert JP, Sens MA, *et al*. Isoform-specific expression of metallothionein mRNA in the developing and adult human kidney. *Toxicol Lett* 1996;85:17–27.
26. Nicassio F, Bianchi F, Capra M, *et al*. A cancer-specific transcriptional signature in human neoplasia. *J Clin Invest* 2005;115:3015–3025.
27. Tai SK, Tan OJ, Chow VT, *et al*. Differential expression of metallothionein 1 and 2 isoforms in breast cancer lines with different invasive potential: identification of a novel nonsilent metallothionein-1H mutant variant. *Am J Pathol* 2003;163:2009–2019.
28. Burger H, Foekens JA, Look MP, *et al*. RNA expression of breast cancer resistance protein, lung resistance-related protein, multidrug resistance-associated proteins 1 and 2, and multidrug resistance gene 1 in breast cancer: correlation with chemotherapeutic response. *Clin Cancer Res* 2003;9:827–836.
29. Detre S, Saclani JG, Dowsett M. A 'quickscore' method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. *J Clin Pathol* 1995;48:876–878.
30. UK Committee on Cancer Research. UKCCCR guide lines for the welfare of animals in experimental neoplasia. *Br J Cancer* 1988;58:109–113.
31. Nartey N, Cherian MG, Banerjee D. Immunohistochemical localization of metallothionein in human thyroid tumors. *Am J Pathol* 1987;129:177–182.
32. Finn SP, Smyth P, Chill S, *et al*. Expression microarray analysis of papillary thyroid carcinoma and benign thyroid tissue: emphasis on the follicular variant and potential markers of malignancy. *Virchows Arch* 2007;450:249–260.
33. Liu D, Liu Z, Condouris S, *et al*. BRAF V600E maintains proliferation, transformation, and tumorigenicity of BRAF-mutant papillary thyroid cancer cells. *Clin Endocrinol Metab* 2007;92:2264–2271.