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HMGA2 mRNA expression correlates with the malignant phenotype in human thyroid neoplasias

Gennaro Chiappetta[a], *Angelo Ferraro*[b], *Emilia Vuttariello*[a], *Mario Monaco*[a], *Francesca Galdiero*[a], *Veronica De Simone*[a], *Daniela Califano*[a], *Pierlorenzo Pallante*[b], *Gerardo Botti*[a], *Luciano Pezzullo*[a], *Giovanna Maria Pierantoni*[b], *Massimo Santoro*[b], *Alfredo Fusco*[b,c,*]

[a]*Istituto Nazionale dei Tumori, Fondazione Pascale, via Mariano Semmola, 80131 Naples, Italy*
[b]*Dipartimento di Biologia e Patologia Cellulare e Molecolare c/o Istituto di Endocrinologia ed Oncologia Sperimentale del CNR, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli "Federico II", via Pansini, 5, 80131, Naples, Italy*
[c]*NOGEC (Naples Oncogenomic Center)-CEINGE, Biotecnologie Avanzate, via Comunale Margherita, 482, 80145, Naples, Italy*

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ABSTRACT

We have analysed the expression of the HMGA2 gene in a panel of normal and neoplastic thyroid tissues by immunohistochemistry and quantitative RT-PCR. HMGA2 protein was detectable in four out of 21 follicular carcinomas, 30 out of 45 papillary carcinomas, and 11 out of 12 undifferentiated carcinomas. As far as follicular thyroid adenomas are concerned, only three cases of the 31 analysed showed HMGA2 protein expression, whereas it was absent in seven normal thyroid tissues and in 12 hyperplastic nodules. Quantitative RT-PCR showed that almost all the papillary thyroid carcinomas and 13 out of 16 follicular thyroid carcinomas express much higher HMGA2 specific mRNA levels in comparison to normal thyroids and adenomas. Therefore, our data support the quantitative RT-PCR analysis of HMGA2 expression, rather than immunohistochemistry, as a powerful tool for the diagnosis of thyroid neoplasias.

1. Introduction

The high mobility group A (HMGA) proteins are architectural chromatin proteins. They bind the minor groove of AT-rich DNA sequences through three short basic repeats, called 'AT-hooks', located at the N-terminal region of the proteins. By interacting with the transcription machinery, HMGA proteins alter the chromatin structure and, thereby, regulate the transcriptional activity of several genes either enhancing or suppressing the ability of more usual transcriptional activators and repressors. The HMGA family is comprised of three proteins: HMGA1a, HMGA1b and HMGA2. The first two proteins are products of the same gene, *HMGA1*, generated through an alternative splicing mechanism. The HMGA1a, HMGA1b and HMGA2 proteins (previously HMGI, HMGY and HMGI-C, respectively) are composed of 107, 96 and 108 aminoacid residues, respectively.[1–3]

HMGA proteins are expressed at a high level during embryogenesis,[4,5] while their expression becomes low or undetectable in adult tissues. HMGA proteins represent a unique example of genes being implicated in both benign

* *Corresponding author*: Address: Dipartimento di Biologia e Patologia Cellulare e Molecolare c/o Istituto di Endocrinologia ed Oncologia Sperimentale del CNR, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli "Federico II", via Pansini, 5, 80131, Naples, Italy. Tel.: +39 081 3737857/7463749; fax: +39 081 3737808.
E-mail addresses: afusco@napoli.com, alfusco@unina.it (A. Fusco).
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and malignant neoplasias, but with different mechanisms. In fact, rearrangements of these genes represent a feature of most benign mesenchymal tumours.[6] Conversely, HMGA overexpression is a common event in experimental and human malignancies and is also required for the appearance of the neoplastic phenotype.[7–9] Indeed, HMGA protein expression was detected in pancreas,[10,11] thyroid,[12,13] colon,[14,15] breast,[16] liver,[17] lung,[18] ovary,[19] prostate,[20] squamous carcinomas of the oral cavity,[21] and head and neck tumours,[22] but not in the respective normal tissues, and HMGA protein expression represents a poor prognostic index since their overexpression often correlates with reduced survival and with metastasis.[16,18]

As far as the analysis of the HMGA1 expression in thyroid neoplasias is concerned, HMGA1 protein was detectable in 18 out of 19 follicular carcinomas (FTC), 92 out of 96 papillary carcinomas (PTC), and 11 out of 11 anaplastic carcinomas (ATC), but in only one out of 20 hyperplastic nodules, 44 out of 200 follicular adenomas (FTA), and 0 out of 12 normal tissue samples. Moreover, a correlation between the HMGA1 expression levels and the aggressiveness of the tumours was found, since the highest expression was observed in the anaplastic histotype, which represents one of the most aggressive and invariably lethal tumour histotypes in mankind. Therefore, the correlation between HMGA1 expression and the diagnosis of carcinoma was highly significant ($P < 0.0001$).[13] However, there are no data about the expression of HMGA2 in thyroid neoplasias even though studies *in vitro* clearly demonstrated the association of the HMGA2 expression with the ability of the transformed cells to grow in agar and induce tumours in athymic mice.[7]

In our studies, we have analysed the expression of the HMGA2 gene in a series of normal and neoplastic thyroid samples by immunohistochemistry and quantitative RT-PCR. HMGA2 protein was detectable in four out of 21 FTCs, 30 out of 45 PTCs, and 11 out of 12 ATCs. As far as FTAs are concerned, only three cases of the 31 analysed showed HMGA2 protein expression, whereas it was absent in seven normal thyroid tissues and in 12 hyperplastic nodules. Quantitative RT-PCR essentially confirmed the immunohistochemical data for FTAs and ATCs, whereas almost all the papillary thyroid carcinomas and 13 out of 16 follicular thyroid carcinomas expressed much higher HMGA2 specific mRNA levels in comparison to normal thyroids and adenomas. Interestingly, among the FTCs and PTCs positive for RT-PCR, there were 12 FTCs and 10 PTCs found negative by immunohistochemical analysis.

2. Materials and methods

2.1. Collection of thyroid tissue samples

A total of 128 thyroid tissue specimens consisting of seven normal thyroid tissues, 12 hyperplastic lesions, 31 follicular adenomas, 21 follicular carcinomas, 45 papillary carcinomas and 12 anaplastic carcinomas were collected at the 'Istituto Nazionale dei Tumori di Napoli' (Naples, Italy) from patients undergoing surgery for thyroid cancer. The FTCs included eight minimally and 13 widely invasive cases. The PTCs include 28 classic cases, 12 follicular variants and six oxyphilic cell types. Informed consent for the scientific use of biological material was obtained from all patients.

All tissue samples were fixed immediately after surgical removal in 4% paraformaldehyde in phosphate-buffered saline (PBS) w/v. The criteria for inclusion in the study were that the routinely processed paraffin blocks were suitable for immunohistochemistry and adequate clinical information.

2.2. Immunohistochemical analysis

Paraffin sections (5–6 μm) were deparaffinised, placed in a solution of absolute methanol and 0.3% hydrogen peroxide v/v for 30 min and then washed in PBS before immunoperoxidase staining. The slides were subsequently incubated with biotinylated goat anti-rabbit/anti-mouse IgG for 20 min (Dako LSAB2 System) and then with streptavidin horseradish peroxidase for 20 min. For immunostaining the slides were incubated in diaminobenzidine (DAB-DAKO) solution containing 0.06 mM DAB and 2 mM hydrogen peroxide in 0.05% PBS v/v pH 7.6 for 5 min. After chromogen development, the slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips using a permanent mounting medium (Permount). Micrographs were taken on Kodak Ektachrome film with a photo Zeiss system. For HMGA2 immunohistochemistry, antibodies (2 μg/ml) raised against the recombinant HMGA2 protein were used.[7] The specificity of the reaction was validated by the absence of staining when carcinoma samples were stained with antibodies pre-incubated with the peptide against which the antibodies were raised (data not shown). Similarly, no positivity was observed when tumour samples were stained with a pre-immune serum (data not shown).

2.3. Statistical analysis

The Fisher's exact test was used to analyse differences of HMGA2 expression among histological types of thyroid tissues.

2.4. Protein extraction, western blotting

Protein extraction and western blotting procedure were carried out as reported elsewhere.[8,9] Membranes were incubated with a primary antibody raised against the HMGA2 protein for 60 min (at room temperature).[7] To ascertain that equal amounts of protein were loaded, the western blot was incubated with antibodies against the γ-tubulin protein (Sigma). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:3000) for 60 min (at room temperature) and the reaction was detected with a western blotting detection system (ECL) (GE Healthcare).

2.5. RNA extraction, cDNA preparation and quantitative Reverse Transcription (qRT)-PCR

Total RNA isolation from human tissues was performed with Trizol (Invitrogen) according to the manufacturer's instructions. RNA was extracted from fresh specimens after pulverising the tumours with a stainless steel mortar and pestle that were chilled on dry ice. The integrity of the RNA was assessed

by denaturing agarose gel electrophoresis. 1 μg of total RNA of each sample was reverse-transcribed with the QuantiTect Reverse Transcription (QIAGEN group) using an optimised blend of oligo-dT and random primers according to the manufacturer's instructions.

To ensure that RNA samples were not contaminated with DNA, negative controls were obtained by performing the PCR on samples that were not reversed-transcribed but otherwise identically processed.

To design a qPCR assay we used a Human ProbeLibrary™ system (Exiqon, Denmark). Briefly, using locked nucleic acid (LNA™) technology,[23,24] Exiqon provides 90 human prevalidate TaqMan probes of only 8–9 nucleotides that recognise 99% of human transcripts in the RefSeq database at NCBI.[25] The free ProbeFinder assay design software, which is an integrated part of the package, is available on the web site www.probelybrary.com. All fluorigenic probes were dual-labelled with FAM at the 5′ end and with a black quencher at the 3′ end.

We chose the best probe and primers pair to amplify a fragment for real-time PCR of HMGA2 mRNA, entering its accession number (NM_003484) on the assay design page of the ProbeFinder software. ProbeFinder generated an intron-spanning assay identifying the exon-exon boundaries within the submitted transcript.[26] Based on these data, the software provided us with various solutions. We chose an amplicon of 77 nucleotides scattered among first and second exon. The number of the probe was 'human 38' (according to the numbering of Exiqon's Human ProbeLibrary kit) and the primer sequences were: *HMGA2 forward* 5′-*gcgcctcagaagagaggac*-3′; *HMGA2 reverse* 5′-*ggtctcttaggagagggctca*-3′.

The same procedure was used to choose both probe and primers for housekeeping gene glucose-6-phosphate dehydrogenase (*g6pd*), accession number X03674. The ProbeFinder provided us with various solutions for *g6pd*, as well as HMGA2 transcript. We opted for an amplicon of 106 nucleotides scattered among third and fourth exon. The number of the probe was 'human 05' and the primer sequences were: *g6pd* forward 5′-acagagtgagcccttcttcaa-3′; *g6pd* reverse 5′-ggaggctgcatcatcgtact-3′. Relative Quantitative TaqMan PCR was performed in Chromo4 Detector, MJ Research in 96-well plates using a final volume of 20 μl. For PCR we used 8 μl of 2,5x RealMasterMix™ Probe ROX (Eppendorf AG, Germany) 200 nM of each primer, 100 nM probe and cDNA generated from 50 ng of total RNA. The conditions used for PCR were 2 min at 95 °C and then 45 cycles of 20 sec at 95 °C and 1 min at 60 °C. Each reaction was performed in duplicate. To calculate the relative expression levels we used the $2^{-\Delta\Delta CT}$ method.[27]

3. Results

3.1. Immunohistochemical detection of HMGA2 proteins in normal and neoplastic thyroid tissues

We have evaluated the expression of the HMGA2 proteins in 128 thyroid tissue specimens (seven normal thyroid tissues, 12 hyperplastic lesions, 31 follicular adenomas, 21 follicular carcinomas, 45 papillary carcinomas and 12 anaplastic carcinomas) by immunohistochemical analysis using antibodies raised versus the N-terminal region of the HMGA2 proteins. These antibodies, as already reported in the past,[7,28] are not able to detect the product of the other member of the HMGA family that is the *HMGA1* gene. The results of this analysis are summarised in Table 1. There was no staining in the normal thyroid tissues and hyperplastic nodules, and only three out of 31 follicular adenomas (9.7%) were positive for HMGA2 staining. Conversely, staining was intense in almost all the anaplastic carcinomas (91.6%), in 66.7% of papillary carcinomas and in 19.1% of follicular carcinomas. As shown in Table 1, no statistically significant differences were observed in HMGA2 staining between FTAs and FTCs ($p = 0.420488$).

In Fig. 1 we show some representative cases of the immunohistochemical analysis. In Panels A, B and C we can observe that no expression of the HMGA2 protein is detectable in normal thyroid, goiter and FTA respectively. In contrast, in Panels D, E and F, positive FTC, PTC and ATC samples are shown: a clear nuclear staining, due to the expression of the HMGA2 protein, can be appreciated. To further validate the immunohistochemical data and the specificity of the antibody, we also performed a western blot analysis of representative samples. As shown in Fig. 2, a band of 17 kd corresponding to the HMGA2 protein was found in the PTCs P6, P16, P23 and P30 (which were also positive for immunohistochemistry). Conversely, HMGA2 was not detected in normal thyroid, in FTA A4, FTC F12 or PTC P9 (which did not show immunostaining - as we show below, FTC F12 and PTC P9

Table 1 – Analysis of HMGA2 protein expression in normal and neoplastic thyroid tissues by immunohistochemistry

Histological type of thyroid tissues	N° of positive cases/ number of cases analysed	Percent of HMGA2 positive cells			
		1+	2+	3+	4+
Normal thyroid	0/7				
Goiters	0/12				
Adenomas	3/31	1	2		
Papillary carcinomas	30/45	5	7	11	7
Follicular carcinomas	4/21	3	1		
Anaplastic carcinomas	11/12		1	6	4

The proportion of malignant cells positively stained was scored from 0 to 4: (0, no positive cells; 1+, <10% of positive cells; 2+, 11–50% of positive cells; 3+, 51–75% of positive cells; and 4+, 76–100% of positive cells).

The Fisher's exact test was used to analyse differences of HMGA2 staining among thyroid tissue histological types. PTCs plus FTCs plus ATCs versus normal tissues and goiters $p < 0.001$; PTCs plus FTCs plus ATCs versus adenomas $p < 0.001$. PTCs versus normal thyroid plus goiters $p < 0.001$; FTCs versus adenomas $p = 0.420488$; FTCs versus normal thyroids plus goiters $p = 0.107900$.

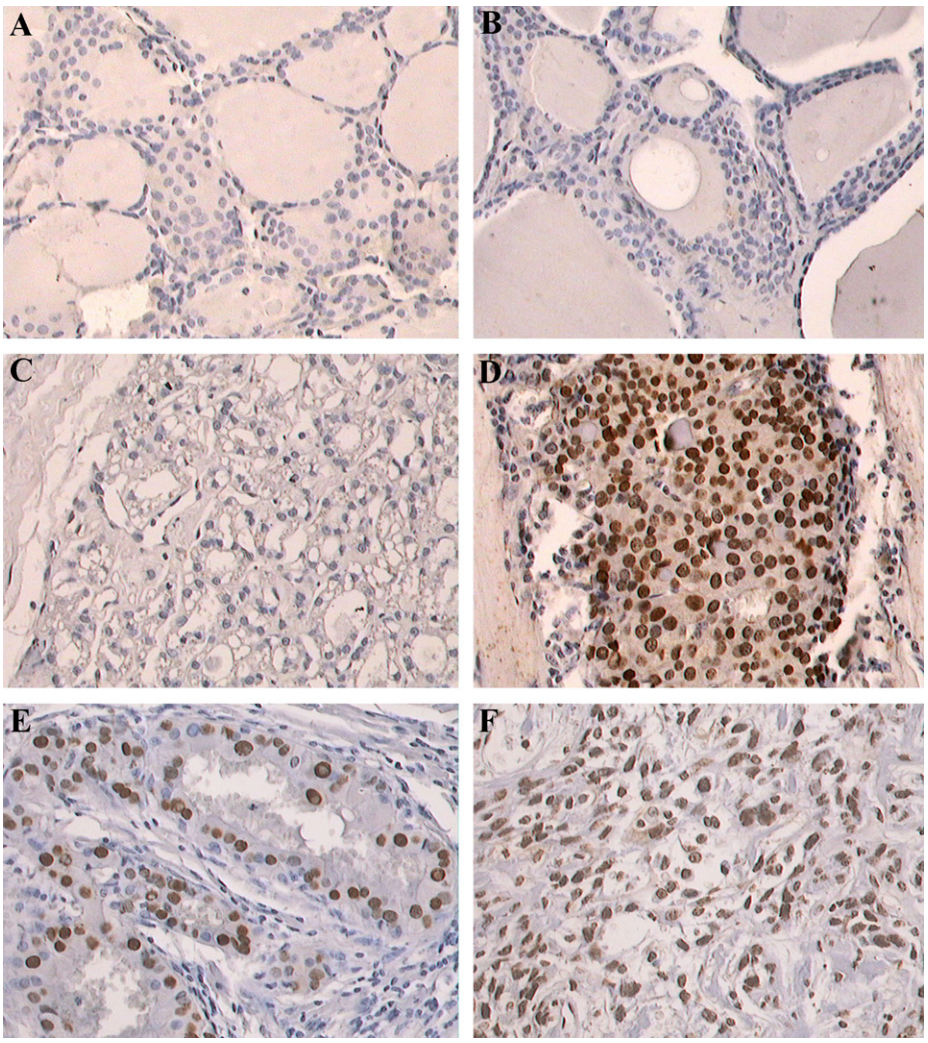

Fig. 1 – Immunohistochemical analysis of HMGA2 protein expression in benign and malignant thyroid tissues. Paraffin sections from hyperplastic and neoplastic thyroid tissues were analysed by immunohistochemistry using antibodies raised against a specific HMGA2 peptide. (A) Immunostaining of a normal thyroid (200×). No immunoreactivity was observed. (B) Immunostaining of a goiter (100×). No immunoreactivity was observed. (C) Immunostaining of a thyroid adenoma (200×). No immunoreactivity was observed. (D) Immunostaining of a thyroid follicular carcinoma (200×). A nuclear staining was observed. (E) Immunostaining of a thyroid papillary carcinoma (200×). A nuclear staining was observed. (F) Immunostaining of an anaplastic thyroid carcinoma (200×). Also in this case, a nuclear positivity was observed.

were confirmed positive for HMGA2 RNA expression by quantitative RT-PCR). It is worth noting that no correlation was found in the follicular adenomas between HMGA2 positivity and any histological feature (e.g. micro- versus macro-follicular) or clinical behaviour. Equally, no correlation was found between the PTC samples negative for HMGA2 expression and histological and clinical parameters of the patients.

3.2. *Quantitative RT-PCR analysis of HMGA2 mRNA expression*

To quantitate the levels of the HMGA2 gene expression and validate the immunohistochemical data we also analysed 70 thyroid samples (six normal thyroid tissues, seven follicular adenomas, 16 follicular carcinomas, 37 papillary carcinomas and four anaplastic carcinomas) for the specific HMGA2 mRNA levels by a quantitative RT-PCR assay. All of these samples were also analysed for HMGA2 by immunohistochemistry.

The results, summarised in Table 2 and shown in Fig. 3, present some discrepancies with the immunohistochemical findings. In fact, FTCs, at odds with the immunohistochemical data, showed in 13 out of 16 cases a great increase in HMGA2 mRNA expression with a fold change of 113.37, with respect to a pool of six normal thyroid tissues. Interestingly, 12 of these FTCs were negative at the immunohistochemical analysis. Conversely, follicular adenomas, apart from one case that also scored positive at the immunohistochemical assay, show expression values not significantly different from normal thyroids. It is important to note that statistical analysis of the qRT-PCR data showed that differences in HMGA2 mRNA expression between FTAs and FTCs were significant ($p = 0.004944$).

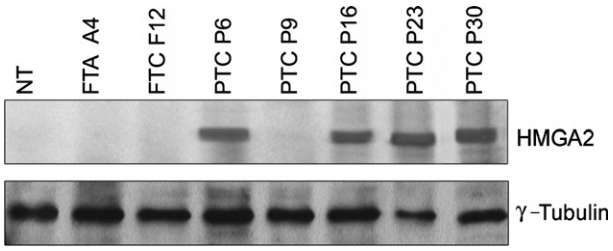

Fig. 2 – Analysis of HMGA2 expression in normal and neoplastic thyroid tissues by Western blot. Normal thyroid, FTA A4, FTC F12 and PTC P9 were also negative for HMGA2 protein expression by immunohistochemistry, whereas PTC P6, P16, P23 and P30 showed positive HMGA2 immunostaining. Blot against γ-tubulin has been performed as control for equal protein loading. NT = normal thyroid; FTA = follicular thyroid adenoma; FTC = follicular thyroid carcinoma; PTC = papillary thyroid carcinoma.

Table 2 – Quantitative RT-PCR analysis of HMGA2 mRNA expression in neoplastic thyroid tissues in comparison with the normal thyroid tissue

Histological type of thyroid tissues	N° of positive cases/number of cases analysed
Adenomas	1/7
Papillary carcinomas	34/37
Follicular carcinomas	13/16
Anaplastic carcinomas	4/4

We have considered positive the cases where the expression of the HMGA2 specific mRNA showed a fold-change increase higher than 4 compared to a pool of six normal thyroid tissues.
Fisher's exact test was used to analyse HMGA2 mRNA expression among thyroid tissue histological types. PTCs plus FTCs plus ATCs versus adenomas $p = 0.000079$. PTCs versus adenomas $p = 0.000078$. FTCs versus adenomas $p = 0.004944$.

As far as ATCs and PTCs are concerned, they showed in most of the cases a great increase in HMGA2 mRNA expression with an average fold change of 199.96 for ATCs and 322.07 for PTCs in comparison to normal thyroids. However, also for PTCs, ten cases, scored negative at the immunohistochemical assay, showed a high HMGA2 mRNA expression.

4. Discussion

In this study we have analysed the expression of HMGA2 both at protein (by immunohistochemistry) and mRNA level (by Real Time RT-PCR). The results described here show a discrepancy between RNA and protein levels as far as the papillary and follicular carcinomas are concerned. In fact, both immunohistochemistry and RT-PCR do not show any significant expression of the HMGA2, as expected, in normal thyroids and adenomas (apart from a couple of samples), whereas a large number of PTCs and almost all the ATCs are positive for HMGA2 staining and express high levels of HMGA2 specific mRNAs. In contrast, the RT-PCR analysis shows a significant increase of HMGA2 mRNA level in most of the follicular carcinomas in comparison with the follicular adenomas: even 12 samples that were scored negative by immunohistochemistry showed a drastic increase in HMGA2 mRNA expression. Also, in the case of PTCs, ten samples scored negative by immunohistochemistry were scored positive by the RT-PCR analysis.

The reasons for this discrepancy are not clear. It would be reasonable to assume that some post-transcriptional mechanisms may be involved, either inhibiting the HMGA2 mRNA translation or affecting the HMGA2 protein degradation. In some FTCs and PTCs we could hypothesise an induction of some microRNAs (miRs) that have HMGA2 as a target. It has been shown recently that the HMGA2 protein is a target of the miR let-7: [29] preliminary results of our group exclude a role of a let-7 on the HMGA2 protein expression in PTCs and

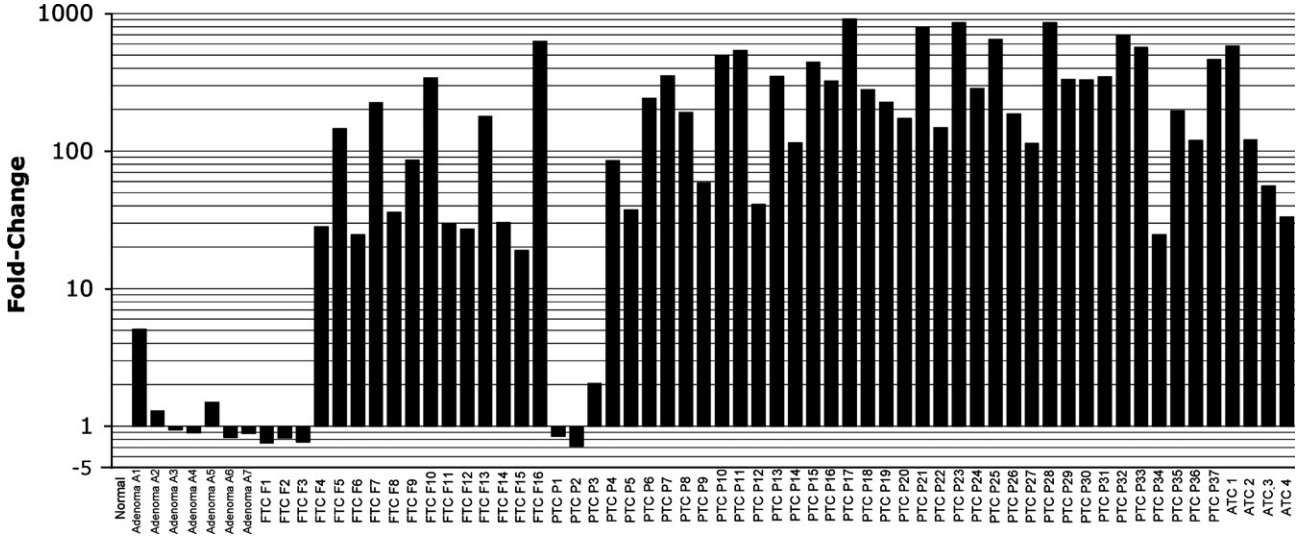

Fig. 3 – Quantitative RT-PCR analysis of HMGA2 expression. Quantitative RT-PCR analysis was performed on a panel of follicular adenoma (FTA), follicular carcinoma (FTC), papillary carcinoma (PTC) and anaplastic thyroid carcinoma (ATC) samples of human thyroid origin. The Fold Change values indicate the relative change in the expression levels between tumour samples and a pool of six normal thyroid tissues assuming that the value of these normal samples was equal to 1.

FTCs since we have observed a decreased let-7 expression in these tumours. However, we cannot exclude an increased expression of other miRs that might have HMGA2 as a target. Another hypothesis is that the HMGA2 protein levels are, in most of the cases, not detectable by immunohistochemistry since this technique is less sensitive than the quantitative RT-PCR.

It is interesting to observe that the immunohistohemical data obtained using the antibodies versus the HMGA2 protein differ from those already published by our group for HMGA1. In fact, almost all the thyroid carcinoma samples were positive for HMGA1 expression.[13] This might suggest some differences in the regulation of these proteins.

Independent from the factors responsible for the discrepancy of mRNA and protein levels in PTCs and FTCs, the results shown here indicate that the evaluation of the HMGA2 specific mRNA level might be a good tool for the discrimination between follicular adenoma and carcinoma that represents one of the main problems in the diagnosis of thyroid neoplasias. In fact, the statistical evaluation of the HMGA2 mRNA levels indicates a significant difference in HMGA2 expression in benign and malignant neoplasias ($p = 0.000079$).

While this work was in progress, a paper regarding the HMGA2 expression in thyroid neoplastic samples was published.[30] Our results are not only confirmatory of these published data, but they also add some more information about the expression of the HMGA2 protein detected by immunohistochemistry, since only representative cases were shown in this paper. In fact, using this technique there is a significant number of samples that score negative. Therefore, the quantitative RT-PCR analysis could be a preferential method to distinguish between benign and malignant follicular neoplasias. The analysis of a significant number of fine needle aspiration biopsies from adenoma and carcinoma samples by qRT-PCR is required for the perspective of the use of this technique as a routine tool in the diagnosis of thyroid neoplastic disorders. This appears particularly important since some studies do not seem to confirm that the analysis of Galectin-3 expression may represent a clear marker for the differential diagnosis between FTA and FTC.[31,32]

In conclusion, our data support the quantitative RT-PCR analysis, rather than immunohistochemistry, of HMGA2 expression as an elective method to distinguish thyroid malignant lesions from the benign ones.

Conflict of interest statement

None declared.

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