Cell Cycle 14:9, 1471–1475; May 1, 2015; © 2015 Taylor & Francis Group, LLC

HMGA1-pseudogene expression is induced in human pituitary tumors

Francesco Esposito[1], Marco De Martino[1], Daniela D'Angelo[1], Paula Mussnich[1], Gerald Raverot[2,3], Marie-Lise Jaffrain-Rea[4,5], Filippo Fraggetta[6], Jacqueline Trouillas[2,7], and Alfredo Fusco[1,8,*]

[1]Istituto di Endocrinologia ed Oncologia Sperimentale del CNR c/o Dipartimento di Medicina Molecolare e Biotecnologie Mediche; Scuola di Medicina e Chirurgia di Napoli; Università degli Studi di Napoli "Federico II", Naples, Italy; [2]U1028; CNRS UMR 5292; Lyon Neuroscience Research Center; Neuro-oncology and Neuroinflammation Team; Lyon, France; [3]Department of endocrinology; Groupement Hospitalier Est; Hospices Civils de Lyon, Lyon, France; [4]Department of Clinical Applied Sciences and Biotechnology; University of L'Aquila; L'Aquila, Italy; [5]Neuromed; IRCCS, Pozzilli, Italy; [6]Department of Pathology; Ospedale Cannizzaro; Catania, Italy; [7]Centre of pathology; Groupement Hospitalier Est; Hospices Civils de Lyon; Lyon, France; [8]Instituto Nacional de Câncer - INCA; Rio de Janeiro, Brazil

Keywords: HMGA1P6, HMGA1P7, HMGA1, ceRNA, miRNA

Numerous studies have established that High Mobility Group A (HMGA) proteins play a pivotal role on the onset of human pituitary tumors. They are overexpressed in pituitary tumors, and, consistently, transgenic mice overexpressing either the *Hmga1* or the *Hmga2* gene develop pituitary tumors. In contrast with HMGA2, HMGA1 overexpression is not related to any rearrangement or amplification of the *HMGA1* locus in these tumors. We have recently identified 2 *HMGA1* pseudogenes, *HMGA1P6* and *HMGA1P7*, acting as competitive endogenous RNA decoys for *HMGA1* and other cancer related genes. Here, we show that *HMGA1* pseudogene expression significantly correlates with *HMGA1* mRNA levels in growth hormone and nonfunctioning pituitary adenomas likely inhibiting the repression of *HMGA1* through microRNAs action. According to our functional studies, these *HMGA1* pseudogenes enhance the proliferation and migration of the mouse pituitary tumor cell line, at least in part, through their upregulation. Our results point out that the overexpression of *HMGA1P6* and *HMGA1P7* could contribute to increase *HMGA1* levels in human pituitary tumors, and then to pituitary tumorigenesis.

Introduction

The High Mobility Group A (HMGA) proteins are non-histone chromatinic proteins involved in transcriptional regulation of gene expression.[1] The HMGA protein family consists of 3 proteins: HMGA1a, HMGA1b, and HMGA2 encoded by 2 different genes, with the HMGA1 proteins being products of the same gene generated through alternative splicing.[1] HMGA overexpression is a feature of malignant neoplasias and its causal role in cell transformation and cancer progression is supported by many studies.[1-3]

We have already reported several evidences that HMGA proteins act as drivers of human pituitary tumors (PT),[4-8] with both the HMGA proteins overexpressed. However, in these tumors, only the overexpression of HMGA2 is associated to gene rearrangement and amplification following trisomy of chromosome 12.[4] Consistently, it is well known that transgenic mice overexpressing either *hmga1* or *hmga2* develop PT,[5-7] and that the HMGA overexpression is associated to the downregulation of several miRNAs able to target both *HMGA1* and *HMGA2* mRNAs in PT (miR-15, miR-16, miR-23b, miR-26a, miR-34b, miR-130b, miR-196a2, miR-326, miR-432, miR-548c-3p, miR-570, miR-603, and Let-7a).[9-11]

We have recently identified 2 *HMGA1* non-coding pseudogenes, *HMGA1P6* and *HMGA1P7*, having conserved seed matches for miRNAs targeting the *HMGA1* gene. The overexpression of *HMGA1* pseudogene (*HMGA1Ps*) increases HMGA1 protein levels, working as competitive endogenous RNA (ceRNA), thereby inhibiting the suppression of HMGA1 protein synthesis by miRNAs.[12,13] Since the *HMGA1Ps* untranslated regions (UTRs) contain also seed sequences for miRNAs able to target HMGA2, their overexpression leads also to increased HMGA2 protein levels. *HMGA1Ps* also show oncogenic activity by inhibiting apoptosis and increasing cell proliferation and migration.[12,13]

The aim of this study has been to investigate the expression and the role of *HMGA1Ps* in PT.

HMGA1 *and* HMGA1Ps *expression positively correlates in pituitary tumors*

Briefly, we analyzed the expression of *HMGA1Ps* by qRT-PCR in a panel of 41 human PT, including 14 growth hormone (GH) tumors with acromegaly and 27 nonfunctioning pituitary adenomas (NFPA) or gonadotroph FSH-LH tumors detected by immunohistochemistry (IHC). As shown in **Fig. 1** (Panels A and

*Correspondence to: Alfredo Fusco; Email: alfusco@unina.it
Submitted: 12/09/2014; Revised: 02/15/2015; Accepted: 02/15/2015
http://dx.doi.org/10.1080/15384101.2015.1021520

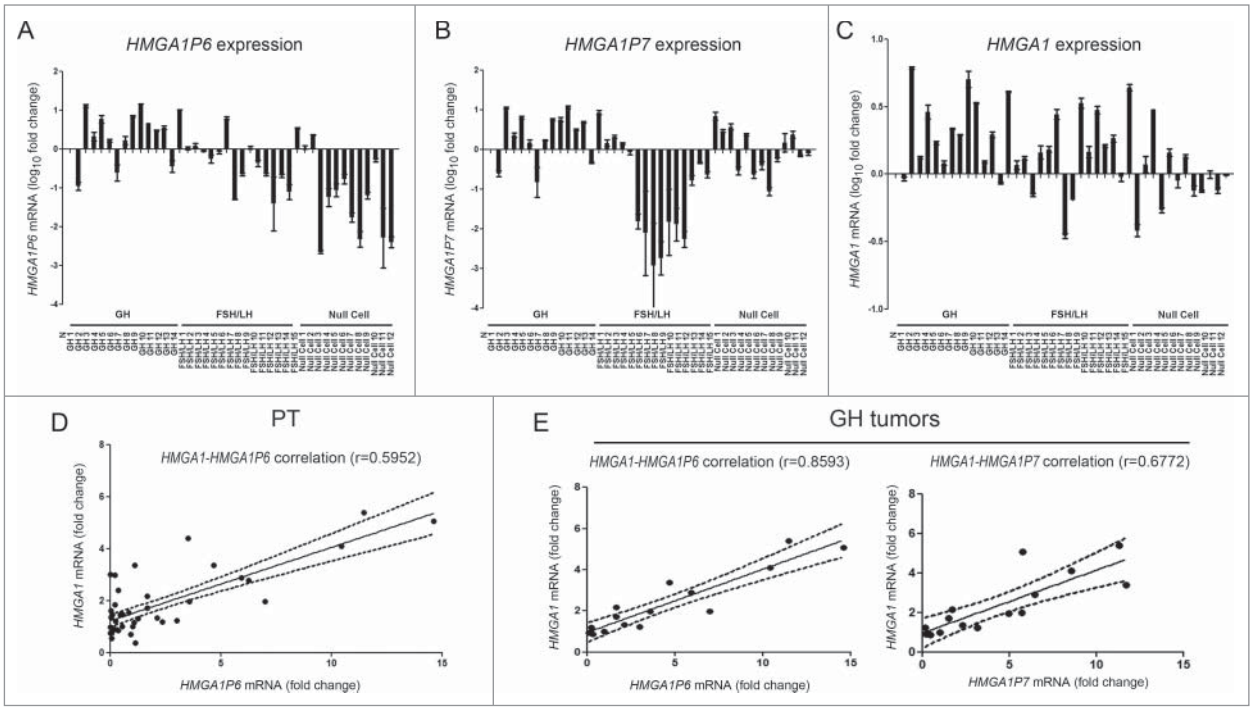

Figure 1. *HMGA1* and *HMGA1Ps* expression positively correlates in pituitary tumors. (**A**) *HMGA1P6*, (**B**) *HMGA1P7* and (**C**) *HMGA1* qRT-PCR analysis in normal pituitary gland (N), GH, FSH/LH and null cell tumors. **(D, E)** The obtained values were then combined for correlation analysis. (**D**) Linear regression of *HMGA1* versus *HMGA1P6* in the whole series of PT. (**E**) Linear regressions of *HMGA1* vs. *HMGA1P6* (left panel) and *HMGA1* (right panel) versus *HMGA1P7* in GH tumors.

B), *HMGA1P6* and *HMGA1P7* were differently expressed with regard to the IHC type, when compared with the normal pituitary gland. To verify whether the 2 *HMGA1Ps* may also function as ceRNAs in the regulation of *HMGA1* mRNA levels in PT, we analyzed *HMGA1* expression in these same samples (**Fig. 1C**).

As indicated in **Figure 1**, Panel D, a significant linear correlation was found between *HMGA1* and *HMGA1P6* expression ($r = 0.5952$, $P < 0.0001$), suggesting that these genes are co-regulated, while there is no correlation between *HMGA1* and *HMGA1P7* expression in the whole tumor series. However, further analysis of *HMGA1* and *HMGA1Ps* co-regulation disclosed some differences according to tumor type. Indeed, in the subset of GH tumors, *HMGA1* strongly correlated with *HMGA1P6* expression ($r = 0.8593$, $P < 0 .0001$), and also with *HMGA1P7* expression ($r = 0.6772$, $P < 0.0001$) (**Fig. 1E**). Taken together, these data strongly support the hypothesis that *HMGA1Ps* could act as ceRNAs in PT and represent a novel potential mechanism of *HMGA1* upregulation in these tumors, in particular in GH tumors with acromegaly.

***HMGA1Ps* expression increases AtT20 cell proliferation and migration acting as ceRNAs for HMGA1**

Subsequently, we examined the ability of *HMGA1P6* and *HMGA1P7* to function as a decoy for *HMGA1*-targeting miRNAs in the pituitary cell line AtT20. **Figure 2**, Panel A, confirms successful transfection of both genes. As shown in **Figure 2**, Panel B, overexpression of either *HMGA1P6* or *HMGA1P7* was associated with HMGA1 protein overexpression. As expected from our previously results,[12] overexpression of *HMGA1P6* or *HMGA1P7* reduced the effects exerted by miRNA on the HMGA1 levels (**Fig. 2B**). Then, to evaluate the functional consequences of *HMGA1P6* and *HMGA1P7* overexpression in PT, we investigated their role in cellular proliferation, and migration in AtT20 cells. To this aim, AtT20 cells were transfected with the *HMGA1P6, HMGA1P7*, and the control vector, and counted each day for 9 d. **Figure 2C**, shows that the growth rate of AtT20 following the transfection of the *HMGA1Ps* was higher compared with the cells transfected with the control vector. Finally, we carried out cell migration assays in the same *HMGA1Ps* overexpressing cells especially considering that HMGA1 promotes cell migration.[1] As expected from the increased HMGA1 protein levels in the pseudogene-transfected cells,[12] cell migration was significantly higher in AtT20 cells overexpressing *HMGA1P6* or *HMGA1P7* than in control cells (**Fig. 2D**).

Discussion

The critical role of High Mobility Group A proteins on the onset of human PT has been widely accepted. In fact, the overexpression of both *HMGA* genes in PT has been reported by numerous studies,[4-8] and consistently the development of GH-PRL tumors is a feature of transgenic mice overexpressing either the *Hmga1* or the *Hmga2* gene.[5, 6]

The results reported here indicate that *HMGA1Ps*, which act as miRNA sponges for *HMGA1* genes, also contribute to

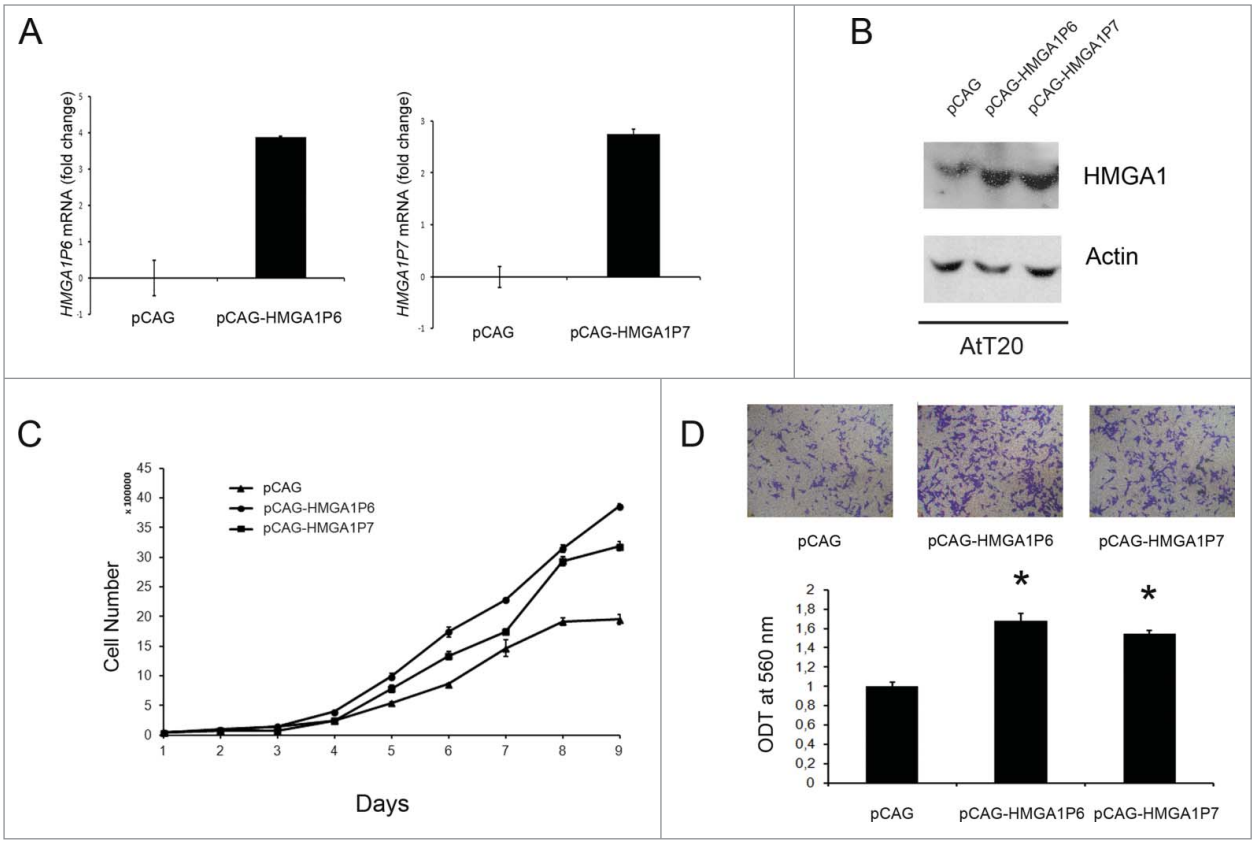

Figure 2. *HMGA1Ps* expression increases AtT20 cell proliferation and migration acting as ceRNAs for HMGA1. (**A**) qRT-PCR analysis of *HMGAP6* and *HMGA1P7* mRNA levels in AtT20 cells transfected with the empty, *HMGA1P6* or *HMGA1P7* vectors. (**B**) Western blot analysis of HMGA1 protein levels from the same samples shown in A. (**C**) AtT20 cell proliferation in *HMGA1P6*- and *HMGA1P7*-transfected cells. (**D**) Cell migration assays of AtT20 cells transfected with *HMGA1P6, HMGA1P7* or with a control vector (Upper panel). Migrated cells were quantified and expressed as mean $\pm$ SD *, $P < 0.05$ (*t* test) (Lower panel).

pituitary tumorigenesis by enhancing pituitary cell proliferation and migration.

Since no rearrangement or amplification of the *HMGA1* locus have been detected in PT, where HMGA1 is overexpressed,[4-8] *HMGA1Ps* overexpression contributes to high HMGA1 protein levels detected in PT together with tumor downregulation of miRNAs targeting *HMGA1* (*miR-15, miR-16, miR-23b, miR-26a, miR-34b, miR-130b, miR-196a2, miR-326, miR-432, miR-548c-3p, miR-570, miR-603, and Let-7a*).[9-11]

Indeed, we found a direct correlation between *HMGA1* and *HMGA1Ps* expression in a series of human PT, in particular in the somatotroph ones. Then, functional assays revealed that *HMGA1P6* and *HMGA1P7* increase cell proliferation and migration in pituitary cell line AtT20. This is in agreement with effects of HMGA1, which accelerates the G1-S transition by increasing E2F1 activity, and enhances cell migration in pituitary cell lines.

Noteworthy, *HMGA1P6, HMGA1P7*, as well as *HMGA1* 3' UTR are potential ceRNAs for other cancer-related genes such as *High Mobility Group A2 (HMGA2)* and *Vascular Endothelial Growth Factor* (*VEGF)* which may further contribute to pituitary tumorigenesis.

In conclusion, the results reported here clearly evidence that *HMGA1* pseudogene overexpression contributes to pituitary tumor behavior, thereby disclosing an additional mechanism accounting for the high expression of *HMGA1* (and likely *HMGA2*) in PT. Consequently, these results further support the perspective of an innovative molecular therapy of PT by restoring the expression of miRNAs able to target the *HMGA* genes and/or blocking that of the *HMGA1* pseudogenes.

Methods

Cell culture and transfections

AtT20 cells were maintained in DMEM supplemented with 10% foetal calf serum (Euroclone; Milan, Italy), glutamine and antibiotics. Cells were repeatedly tested with MycoAlert (Lonza; Slough, UK) to ensure that cells were not infected with mycoplasma. Cells were transfected using Lipofectamine plus reagent (Life Technologies Italia; Monza, Italy) according to the manufacturer's instructions. The transfected cells were selected in a medium containing geneticin (Sigma; St. Louis, USA). For each transfection, several geneticin-resistant mass cell populations were isolated and expanded for additional analysis. Transfection efficiency was established for each experiment by evaluating GFP

expression. *HMGA1P6* and *HMGA1P7* expression vectors have been previously described.[12]

Tissue collection and RNA isolation

Surgical samples of PT were obtained from patients operated in 2 centers: Lyon, France (29 tumors - n°1 to n°29) and at the Neuromed Institute, Pozzilli, Italy (12 tumors - n°30 to n°41). Among these, 14 presented with acromegaly and 27 with clinically non-functioning tumors, respectively. According to diagnostic immunohistochemistry with pituitary hormones, immunostaining for GH was confirmed in tumors from acromegalic patients whereas the large majority of clinically non-functioning tumors showed some degree of immunopositivity for FSH and/or LH and the few immunonegative samples were assimilated to gonadotroph tumors. For each tumor, fragments were fixed in the Bouin-Holland fluid or formol and embedded in paraffin for pathological diagnosis, including IHC. Other fragments were immediately frozen and stored at −80°C. We declare that informed consent for the scientific use of biological material was obtained from all patients.

RNA extraction and quantitative reverse transcription PCR

Total RNA was extracted from tissues with Trizol (Life Technologies Italia; Monza, Italy) according to the manufacturer's instructions. For mRNA detection, total RNA was reverse transcribed by using the QuantiTect Reverse Transcription Kit (Qiagen; Valencia, USA), and then Real-time PCR was performed by using Power SYBR Green PCR Master Mix (Applied Biosystems-Life Technologies Italia; Monza, Italy) and the following primers:

HMGA1-Fw 5′-aaggggcagacccaaaaa-3′
HMGA1-Rev 5′-tccagtcccagaaggaagc-3′
HMGA1P6-Fw 5′-gcagacccacaaaactgga-3′
HMGA1P6-Rev 5′-gagcaaagctgtcccatcc-3′
HMGA1P7-Fw 5′-gctccttctcggctcctc-3′
HMGA1P7-Rev 5′-gcttgggcctcttttatgg-3′
G6PD-Fw 5′-acagagtgagcccttcttcaa-3′
G6PD-Rev 5′-ataggagttgcgggcaaag-3′

The $2^{-\Delta\Delta Ct}$ formula was used to calculate the differential gene expression.

Protein extraction, western blotting and antibodies

Protein extraction and Western blotting were performed as before described.[14] Antibodies against the HMGA1 proteins are described elsewhere.[15] Blots were visualized by using the Western blotting detection reagents (Thermo Scientific, Waltham, USA).

Cell migration assay

Cell migration experiments were performed as previously described.[16]

Statistical analysis

Data were analyzed using a 2-sided unpaired Student's t test (GraphPad Prism, GraphPad Software, Inc.). Values of $P < 0.05$ were considered statistically significant. Regression analyses and correlation coefficients were generated using GraphPad Prism, GraphPad Software, Inc.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Mario Berardone for the artwork. We are grateful to E. Jouhanneau, V. Esposito and F. Giangaspero for tumors collection.

Funding

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro-AIRC (IG 11477), P.O.R. Campania FSE 2007-2013 - Progetto CREMe - CUP B25B09000050007, "Progetto di Interesse strategico Invecchiamento (PNR-CNR Aging Program) PNR-CNR 2012–2014", Progetto PON01-02782 "Nuove strategie nanotecnologiche per la messa a punto di farmaci e presidi diagnostici diretti verso cellule cancerose circolanti", CNR Epigenomics Flagship Project "EPIGEN".

References

1. Fusco A, Fedele M. Roles of the HMGA proteins in cancer. Nat Rev Cancer 2007; 7: 899-910; PMID:18004397; http://dx.doi.org/10.1038/nrc2271
2. Pegoraro S, Ros G, Piazza S, Sommaggio R, Ciani Y, Rosato A, Sgarra R, Del Sal G, Manfioletti G. HMGA1 promotes metastatic processes in basal-like breast cancer regulating EMT and stemness. Oncotarget 2013; 4: 1293-308; PMID:23945276
3. Puca F, Colamaio M, Federico A, Gemei M, Tosti N, Bastos AU, Del Vecchio L, Pece S, Battista S, Fusco A. HMGA1 silencing restores normal stem cell characteristics in colon cancer stem cells by increasing p53 levels. Oncotarget 2014; 5: 3234-45; PMID:24833610
4. Pierantoni GM, Finelli P, Valtorta E, Giardino D, Rodeschini O, Esposito F, Losa M, Fusco A, Larizza L. High-mobility group A2 gene expression is frequently induced in non-functioning pituitary adenomas (NFPAs), even in the absence of chromosome 12 polysomy. Endocr Relat Cancer 2005; 12: 867-74; PMID:16322327; http://dx.doi.org/10.1677/erc.1.01049
5. Fedele M, Pierantoni GM, Visone R, Fusco A. Critical role of the HMGA2 gene in pituitary adenomas. Cell Cycle 2006; 5: 2045-8; PMID:16969098; http://dx.doi.org/10.4161/cc.5.18.3211
6. Fedele M, Visone R, De Martino I, Troncone G, Palmieri D, Battista S, Ciarmiello A, Pallante P, Arra C, Melillo RM, et al. HMGA2 induces pituitary tumorigenesis by enhancing E2F1 activity. Cancer Cell 2006; 9: 459-71; PMID:16766265; http://dx.doi.org/10.1016/j.ccr.2006.04.024
7. Fedele M, Fusco A. Role of the high mobility group A proteins in the regulation of pituitary cell cycle. J Mol Endocrinol 2010; 44: 309-18; PMID:20219853; http://dx.doi.org/10.1677/JME-09-0178
8. Palmieri D, Valentino T, De Martino I, Esposito F, Cappabianca P, Wierinckx A, Vitiello M, Lombardi G, Colao A, Trouillas J, et al. PIT1 upregulation by HMGA proteins has a role in pituitary tumorigenesis. Endocr Relat Cancer 2012; 19: 123-35; PMID:22199144; http://dx.doi.org/10.1530/ERC-11-0135
9. Palmieri D, D'Angelo D, Valentino T, De Martino I, Ferraro A, Wierinckx A, Fedele M, Trouillas J, Fusco A. Downregulation of HMGA-targeting microRNAs has a critical role in human pituitary tumorigenesis. Oncogene 2012; 31: 3857-65; PMID:22139073; http://dx.doi.org/10.1038/onc.2011.557
10. D'Angelo D, Palmieri D, Mussnich P, Roche M, Wierinckx A, Raverot G, Fedele M, Croce CM, Trouillas J, Fusco A. Altered microRNA expression profile in human pituitary GH adenomas: down-regulation of miRNA targeting HMGA1, HMGA2, and E2F1. J Clin Endocrinol Metab 2012; 97: E1128-38; PMID:22564666; http://dx.doi.org/10.1210/jc.2011-3482
11. Leone V, Langella C, D'Angelo D, Mussnich P, Wierinckx A, Terracciano L, Raverot G, Lachuer J, Rotondi S, Jaffrain-Rea ML, et al. Mir-23b and miR-130b expression is downregulated in pituitary adenomas. Mol Cell Endocrinol 2014; 390: 1-7; PMID:24681352; http://dx.doi.org/10.1016/j.mce.2014.03.002

12. Esposito F, De Martino M, Petti MG, Forzati F, Tornincasa M, Federico A, Arra C, Pierantoni GM, Fusco A. HMGA1 pseudogenes as candidate proto-oncogenic competitive endogenous RNAs. Oncotarget 2014; 5: 8341-54; PMID:25268743
13. Esposito F, De Martino M, Forzati F, Fusco A. HMGA1-pseudogene overexpression contributes to cancer progression. Cell Cycle 2014; 13: 3636-9; PMID:25483074; http://dx.doi.org/10.4161/15384101.2014.974440
14. Martinez Hoyos J, Ferraro A, Sacchetti S, Keller S, De Martino I, Borbone E, Pallante P, Fedele M, Montanaro D, Esposito F, et al. HAND1 gene expression is negatively regulated by the High Mobility Group A1 proteins and is drastically reduced in human thyroid carcinomas. Oncogene 2009; 28: 876-85; PMID:19060921; http://dx.doi.org/10.1038/onc.2008.438
15. Piscuoglio S, Zlobec I, Pallante P, Sepe R, Esposito F, Zimmermann A, Diamantis I, Terracciano L, Fusco A, Karamitopoulou E. HMGA1 and HMGA2 protein expression correlates with advanced tumour grade and lymph node metastasis in pancreatic adenocarcinoma. Histopathology 2012; 60: 397-404; PMID:22276603; http://dx.doi.org/10.1111/j.1365-2559.2011.04121.x
16. Borbone E, Troncone G, Ferraro A, Jasencakova Z, Stojic L, Esposito F, Horning N, Fusco A, Orlando V. Enhancer of zeste homolog 2 overexpression has a role in the development of anaplastic thyroid carcinomas. J Clin Endocrinol Metab 2011; 96: 1029-38; PMID:21289264; http://dx.doi.org/10.1210/jc.2010-1784