

# Different expression of protein kinase A (PKA) regulatory subunits in normal and neoplastic thyroid tissues

Stefano Ferrero<sup>1,2</sup>, Valentina Vaira<sup>2</sup>, Alessandro del Gobbo<sup>2</sup>, Leonardo Vicentini<sup>3</sup>,  
Silvano Bosari<sup>4,2</sup>, Paolo Beck-Peccoz<sup>5</sup>, Giovanna Mantovani<sup>5</sup>, Anna Spada<sup>5</sup> and Andrea G. Lania<sup>6</sup>

<sup>1</sup>Department of Biomedical, Surgical and Dental Sciences, University of Milan Medical School, <sup>2</sup>Division of Pathology, Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico, <sup>3</sup>Endocrine Surgery Unit, Fondazione IRCCS Ca' Granda – Ospedale Maggiore Policlinico, <sup>4</sup>Department of Pathophysiology and Organ Transplant, University of Milan Medical School, <sup>5</sup>Endocrinology and Diabetology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and <sup>6</sup>Endocrinology Unit, IRCCS Istituto Clinico Humanitas, Rozzano, Milan, Italy

**Summary.** The four regulatory subunits (R1A, R1B, R2A, R2B) of protein kinase A (PKA) are differentially expressed in several cancer cell lines and exert distinct roles in both cell growth and cell differentiation control. Mutations of the *PRKARIA* gene have been found in patients with Carney complex and in a minority of sporadic anaplastic thyroid carcinomas. The aim of the study was to retrospectively evaluate the expression of different PKA regulatory subunits in benign and non benign human thyroid tumours and to correlate their expression with clinical phenotype.

Immunohistochemistry demonstrated a significant increase in PRKAR2B expression in both differentiated and undifferentiated (anaplastic) thyroid tumors in comparison with normal thyroid tissues. Conversely, a significant increase in PRKAR1A expression was only demonstrated in undifferentiated thyroid carcinomas in comparison with normal thyroid tissue and differentiated thyroid tumors. In thyroid cancers without lymph nodal metastases PRKAR1A expression was higher in tumours of more than 2 cm in size (T2 and T3) compared to smaller ones (T1).

In conclusion, our data shows that an increased PRKAR1A expression is associated with aggressive and undifferentiated thyroid tumors.

**Key words:** PKA, Thyroid neoplasia, PRKAR1A, PRKAR2B

## Introduction

Evidence from *in vitro* studies and naturally occurring human diseases indicate that thyroid cell growth is deeply influenced by intracellular cAMP levels (Feliciello et al., 2000; Dremier et al., 2002; Horvath et al., 2009). In particular, mutations of the stimulatory Gs protein gene and TSH receptor leading to the constitutive activation of adenylyl cyclase have been found in a small subset of thyroid toxic adenomas (Moretti et al., 2000). Recently, genetic defects downstream from cAMP production and affecting cAMP degradation have been recognized. In particular, inactivating mutations and variants of phosphodiesterases, i.e. PDE11A, have been implicated in endocrine tumorigenesis while PDE8B polymorphisms have been proposed to be involved in the regulation of TSH levels (Horvath et al., 2006, 2009, 2010a,b).

Recently, genetic defects downstream from cAMP production and affecting PKA complex have been identified in endocrine disorders, associated with benign and malignant neoplasia (Kirschner et al., 2000; Moretti et al., 2000; Sandrini et al., 2002; Porcellini et al., 2003; Lania et al., 2004; Mantovani et al., 2009; Horvath et al., 2010a; Pringle et al., 2012). In mammalian cells there are two types of PKA, PKA1 and PKA2, which are distinguished by 4 different regulatory subunits (R1A, R1B, R2A and R2B) that differ in tissue distribution,

Offprint requests to: Stefano Ferrero MD, Department of Biomedical, Surgical and Dental Sciences, University of Milan Medical School, Milan, Italy- Fondazione IRCCS "Ca' Granda" - Ospedale Maggiore Policlinico, Vai F. Sforza 35. Milano, 20122, Italy. e-mail: stefano.ferrero@unimi.it

subcellular localization and biological properties. Dramatic changes in the proportion of R1 and R2 subunits during ontogenic development, differentiation processes and neoplastic transformation indicate distinct roles for these isoenzymes in growth control. In particular, R1 subunits seem to be primarily involved in cell proliferation while R2 subunits relate to tissue differentiation (Cho-Chung et al., 1995).

Carney complex is most commonly caused by mutations in the *PRKARIA* gene on chromosome 17q23-q24 and inactivating germline mutations of this gene are found in 70% of people with Carney complex.

Few data on the impact of PKA1 and PKA2 activation and R1 and R2 subunits expression on thyroid cell differentiation and growth are available. Experiments on mouse fibroblasts showed that the expression of PRKAR2B was able to confer TSH-cAMP dependent growth (Porcellini et al., 2003). Similarly, the essential role of PRKAR2B on gene transcription and proliferation was confirmed in FRTL5 cells (Sandrini et al., 2002). Analyses on human cancer cell lines, i.e. NPA (melanoma) and ARO (colonic carcinoma) cells, pointed to a possible loss of PRKAR2B expression and an anti-proliferative action of PRKAR1A activation in these systems (Calebiro et al., 2006).

Moreover, a tissue-specific knockout of *PRKARIA* thyroid resulted in hyperthyroid mice that developed follicular thyroid neoplasms, including follicular carcinomas in over 40% of animals (Pringle et al., 2012), and downregulated *PRKARIA* gene showed thyroid findings in Carney complex patients, supporting its role as a candidate tumour suppressor gene (Griffin et al., 2004).

The aim of the study was to retrospectively evaluate the expression of different PKA regulatory subunits in benign and non benign human thyroid tumours and to correlate their expression with clinical phenotype, in order to demonstrate whether PRKAR1A and PRKAR2B can be useful markers of malignancy for thyroid cancer.

## Materials and methods

### Patients

In this study we retrospectively evaluated 6 nodular hyperplasia, 16 follicular adenomas, 12 follicular carcinomas (4 males and 8 females, age 51±11 years), 15 papillary carcinomas (4 males and 11 females, age 49±18 years) as well as 4 undifferentiated (anaplastic) thyroid carcinomas (3 males and 1 female, age 66±9 years). For comparison, 10 normal specimens, collected as far as possible from neoplastic lesions of patients surgically resected for thyroid tumor, were included in the analysis. After surgical resection, all tissues were promptly formalin-fixed and paraffin embedded.

Staging of neoplastic lesions was accomplished according to the last edition of TNM (VII edition, 2009), and patient characteristics are summarized in Table 1. A

paraffin block of the lesion representative of each case was selected. Four sections, 3 micrometers-thick, were cut for standard hematoxylin-eosin and immunohistochemical staining.

### Immunohistochemistry

Specific monoclonal antibodies for PRKAR1A, and PRKAR2B were used (BD Transduction Laboratories, Lexington, United Kingdom) and antigen-antibody detection was performed with the DAKO ChemMate En Vision detection kit (DAKO A/S, Glostrup, Denmark) according to the manufacturer's instructions as previously described (Lania et al., 2004). Sections were stained with 3,3'-diaminobenzidine substrate and quickly counterstained with Meyer hematoxylin. Slides were finally prepared for light microscopy examination. Normal human adrenal tissue was used as positive control, whereas for negative ones either the primary

**Table 1.** Clinical and immunohistochemical characteristics of patients affected with malignant thyroid lesions.

Cases	Age	Sex	TNM	Stage	R1A score	R2B score
FC1	67	F	pT3N0	III	1	0
FC2	49	M	pT3N0	III	6	0
FC3	42	F	pT3N0	I	3	10
FC4	49	F	pT3N0	III	6	0
FC5	52	F	pT3N0	III	6	0
FC6	66	M	pT2N0	II	4	9
FC7	60	M	pT3N0	II	6	9
FC8	40	F	pT3N0	I	1	9
FC9	30	M	pT3N0	I	1	9
FC10	54	F	pT2N0	II	3	9
FC11	59	F	pT2N0	II	1	9
FC12	42	F	pT1N0	I	2	9
PC1	80	M	pT1N0	I	4	0
PC2	35	F	pT1N1	I	6	1
PC3	58	F	pT3N0	III	1	0
PC4	74	F	pT2N0	II	6	0
PC5	70	M	pT3N0	III	2	0
PC6	61	M	pT3N1	III	6	1
PC7	32	F	pT1N1	I	6	0
PC8	52	F	pT1N0	I	1	9
PC9	56	F	pT1N0	I	1	9
PC10	23	F	pT1N0	I	1	9
PC11	43	F	pT3N1	I	1	9
PC12	27	F	pT3N1	I	4	9
PC13	31	F	pT3N0	I	1	9
PC14	42	F	pT3N1	I	2	9
PC15	60	M	pT1N0	I	1	9
AC1	53	M	pT4N3	IV	9	0
AC2	74	M	pT4N4	IV	6	3
AC3	65	M	pT4N4	IV	9	9
AC4	72	F	pT4N4	IV	9	9

PKAR1A and PKAR2B immunoreactivities were graded according to an immunohistochemical score (range from 0 to 9 units) that is the product of the percentage of positive cells for the intensity of the staining (see Material and Methods). FC, follicular carcinoma; PC, papillary carcinoma; AC, anaplastic carcinoma.

## PKA in thyroid neoplasia

antibody was omitted or an unrelated mouse monoclonal antibody was employed. Briefly, PRKAR1A and PRKAR2B immunoreactivities were graded according to an immunohistochemical score (IHC score; range: 0-9 units) that takes into account both the percentage of positive cells and the staining intensity. At least 400 cells in the main representative high power field were considered for each case and the percentage of positive cells was estimated (0-30% = 1; 31-60% = 2; 61-100% = 3). The staining grade varied from 0 to 3, following these criteria: 0 = absence of immunoreactivity, 1 = weak, 2 = medium intensity and 3 = strong reactivity. The IHC score was then computed by multiplying the two values for each sample.

Three authors (SF, VV, ADG) looked at and graded the specimens for all antibodies under study independently. When the authors were discordant, the section was revised and an agreement was achieved.

### Statistical analysis

For statistical analysis, GraphPad Prism 5 software was used. For two samples or group comparison the nonparametric Mann-Whitney U or the Kruskal-Wallis tests were used, respectively. The results are expressed as the mean  $\pm$  SEM. P values less than  $<0.05$  were accepted as statistically significant.

## Results

### PRKAR1A expression

PRKAR1A protein was expressed in all the normal or pathological thyroid samples analyzed, with a general upregulation of PRKAR1A in thyroid adenomas ( $p < 0.05$  by Kruskal-Wallis test) and carcinomas compared to normal gland ( $p < 0.05$  by Kruskal-Wallis test; Fig. 1a).

Specifically, PRKAR1A was overexpressed in either follicular or papillary thyroid carcinomas (average score  $3.3 \pm 0.6$  and  $2.9 \pm 0.5$ , respectively) compared to hyperplasia or follicular adenomas (score average =  $1.3 \pm 0.2$  and  $2.2 \pm 0.2$ , respectively), peaking with the highest staining in undifferentiated carcinomas (mean immunohistochemistry score =  $8.3 \pm 0.7$ ;  $p < 0.0001$  by Kruskal-Wallis test; Fig. 1A). The immunoreactivity for PRKAR1A in all thyroid carcinomas was diffuse and anaplastic carcinomas showed an intense and broad protein expression.

### PRKAR2B expression

Weak or no immunoreactivity for PRKAR2B was found in normal thyroid samples (mean immunohistochemistry score  $0.7 \pm 0.3$ ), and this pattern of immunoreactivity was similar to that observed in nodular hyperplasia (mean immunohistochemistry score  $0.4 \pm 0.2$ ; Fig. 1B). Conversely, this protein was upregulated in benign thyroid tumors and in carcinomas ( $p = 0.005$  by Kruskal-Wallis test; Fig. 1B).

In follicular adenoma samples PRKAR2B was highly expressed with an average immunohistochemistry score of  $6.3 \pm 1$  ( $p < 0.05$  vs normal or hyperplastic thyroid) (Fig. 1B). As for benign tumors, all papillary or follicular carcinomas revealed a generally elevated expression of PRKAR2B subunit (averaged score =  $4.9 \pm 1.1$  and  $6.1 \pm 1.3$ , respectively) although its staining was highly heterogeneous between specimens (Fig. 1B and Table 1).

In anaplastic carcinomas PRKAR2B expression was similar to that observed in both papillary and follicular carcinomas (mean immunohistochemistry score =  $5.2 \pm 2.2$ ) (Fig. 1B, Table 1).

### PKA regulatory subunits expression and clinical phenotype

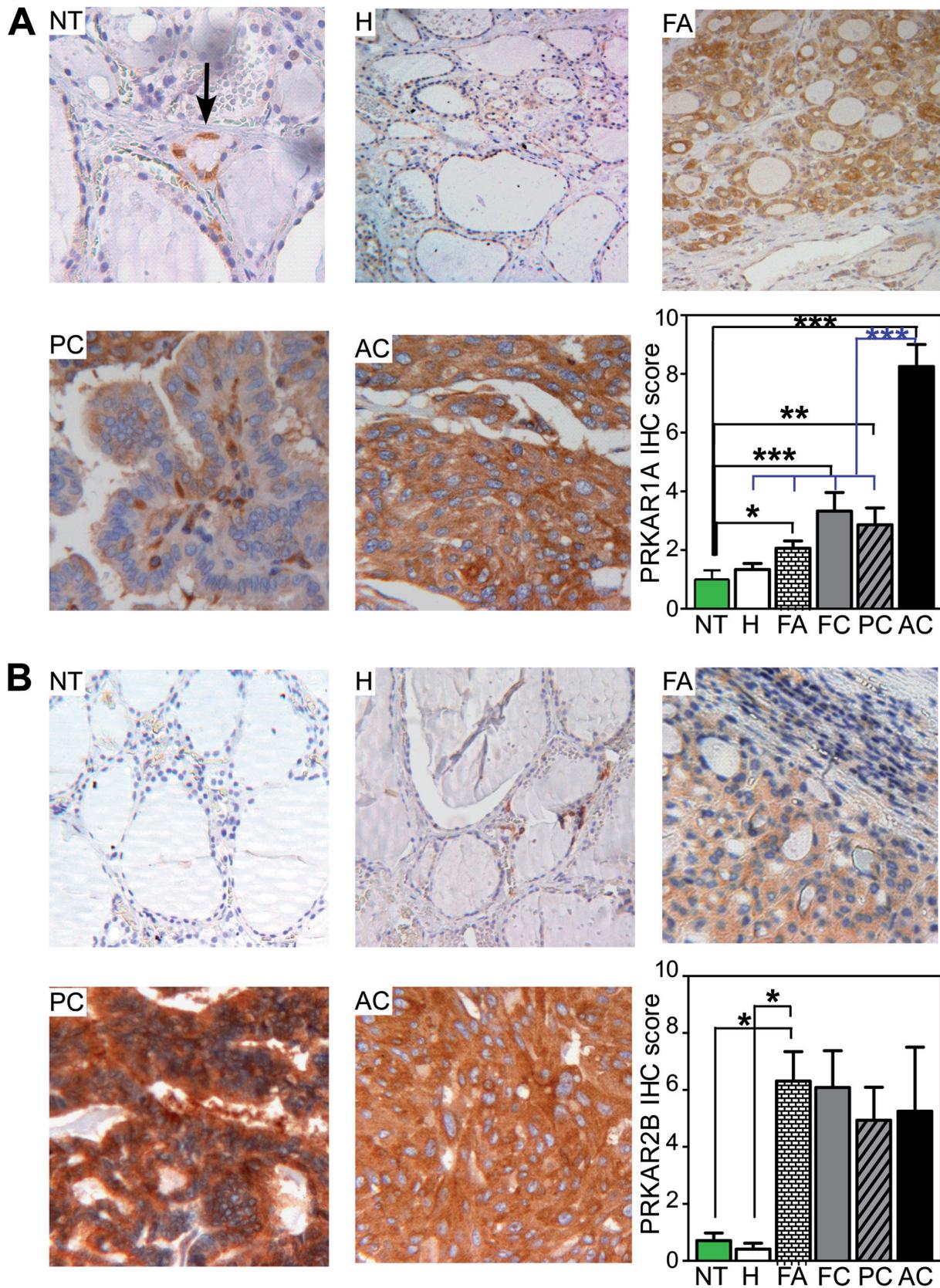
As far as clinical and pathological phenotype in differentiated thyroid carcinomas was concerned, both PRKAR2B and PRKAR1A expression did not correlate with sex or patients' age at presentation. A marginal upregulation of PRKAR1A was observed in papillary or follicular carcinomas with metastatic lymph nodes (average score N0 =  $2.6 \pm 0.4$ , mean score N1 =  $4.2 \pm 0.9$ ; Fig. 2A). Considering only tumors without lymph nodes involvement (N0), a significant reduction of PRKAR1A expression was observed in papillary or follicular carcinoma of less than 2 cm in size (T1) in comparison with the those bigger than 2 cm in size ( $>T1$ ) (mean immunohistochemistry score =  $1.6 \pm 1.8$  vs  $3.5 \pm 1.6$ ;  $p = 0.03$  by Mann-Whitney U test; Fig. 2B). Conversely, PRKAR2B displayed an opposite though not significant expression pattern in the aforementioned categories of thyroid tumours (Fig. 2A,B).

## Discussion

This study describes the expression of the two main regulatory subunits of PKA in a relevant series of neoplastic and non-neoplastic thyroid samples and provides evidence for a specific expression pattern of these subunits in different pathological conditions.

TSH is the key modulator of thyroid cell growth, function and differentiation, most of these effects being mediated by the increase of intracellular cAMP concentration. Although in non-endocrine cells cAMP increases are generally associated with differentiation and growth inhibition, in thyrocytes cAMP pathway mediate proliferative signals. Indeed, the rationale for TSH suppressive post-surgical treatment in patients with thyroid cancers is based on these biological actions of TSH.

The consideration that most cAMP effects occur through the activation of PKA and that the expression of the two regulatory subunits PRKAR1A and PRKAR2B, which are involved in the specific activation of PKA1 and PKA2, varies considerably during tumoral transformation, led us to investigate the relative expression of these subunits in thyroid neoplastic and



**Fig. 1.** Representative immunohistochemistry pictures for PRKAR isoforms 1A (**A**) and 1B (**B**) expression in normal thyroid (NT), nodular hyperplasia (H), follicular adenoma (FA), papillary carcinoma (PC), and anaplastic carcinoma (AC) specimens. The arrow in normal thyroid (**A**) indicates a vessel used as internal positive control. PRKAR1A and PRKAR2B immunoreactivities were graded according to an immunohistochemical score (IHC score, range from 0 to 9 units) that is the product of the percentage of positive cells for the intensity of the staining (see Material and Methods). The quantification of the IHC scores in normal or pathological thyroid samples is provided. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  by Dunns post-test. A: NT, x 20; H, x 4; FA, x 10, PC, AC, x 40. B: NT, H, x 4; FA, x 10; PC, AC, x 40.

PKA in thyroid neoplasia

non-neoplastic tissues. Indeed, the specific role of PKA isoenzymes on thyroid cell proliferation is still controversial and data supporting a major impact of PKA1 or PKA2 mostly derive from studies carried out on thyroid cell lines (Calebiro et al., 2006; Cho-Chung et al., 1995; Porcellini et al., 2003).

The present study showed that normal and hyperplastic thyroid tissues were characterized by a low expression of PRKAR1A and PRKAR2B, whereas both subunits were significantly overexpressed in adenomas and carcinomas. However, the pattern of expression of PRKAR1A was strongly dependent on the histotypes, being higher in anaplastic carcinomas in comparison to differentiated carcinomas and hyperplastic or normal thyroid tissues. Moreover, it is worth noting that in the present series increased PRKAR1A expression was found to be significantly associated with large carcinomas (T2, T3) compared with T1 carcinomas, without considering anaplastic carcinomas that were all T4 stage. Conversely, the increase in PRKAR2B expression did not correlate with cell differentiation, being similar in follicular adenomas and follicular, papillary and anaplastic cancers.

Taken together these data it is tempting to speculate that the increased levels of PRKAR1A and PRKAR2B detected in thyroid neoplasia, and in particular increased levels of PRKAR1A in anaplastic cancers, might result in abnormal PKA activation. Data concerning PKA activity and cAMP dependent signalling in human thyroid cancers are few and mainly focused on cancers bearing inactivating mutations of PRKAR1A gene leading to premature termination and loss of the predicted protein (Cho-Chung et al., 1995). Therefore,

additional functional studies are needed to understand the biological impact of increased PRKAR1A expression on thyroid tumorigenesis.

We found that normal thyroid tissues taken at surgery as far as possible from neoplasia express low levels of both PKA regulatory subunits. In particular, absent or weak immunoreactivity for PRKAR2B was detected in non-neoplastic tissues, while a weak PRKAR1A immunoreactivity was detectable in either normal or hyperplastic thyroid samples.

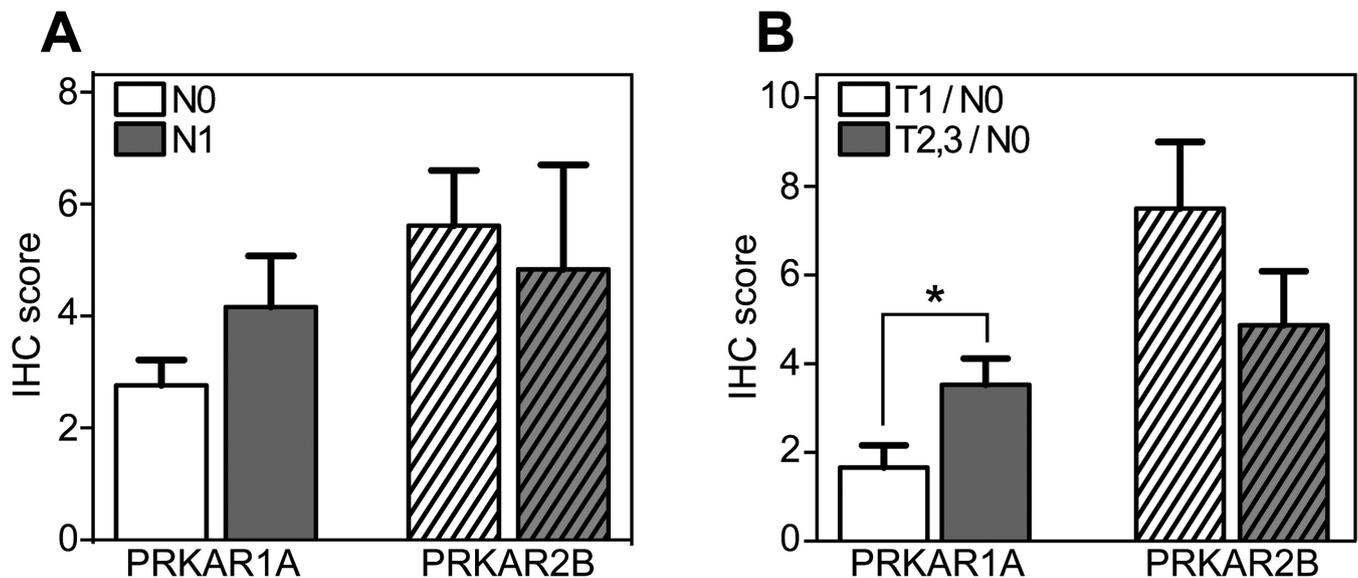
Interestingly our study evidences a significant increase in R1A subunit expression in neoplastic thyroids, including anaplastic carcinomas.

More importantly, higher R1A expression was significantly related to larger tumors in all the samples analyzed and this pattern of immunoreactivity was comparable to nodular hyperplasia.

When considering benign follicular adenomas, PRKAR1A showed a slight but significant increase in immunoreactivity compared to normal thyroid specimens. Moreover, PRKAR2B was highly expressed and was higher in comparison with PRKAR1A.

In this study, we demonstrated that PRKAR1A expression might be an index of malignancy for thyroid cancer, in particular in undifferentiated (anaplastic) tumors and in cases where tumor size is more than 2 cm, and that PRKAR2B expression, even if it did not correlate with cell differentiation, might be a useful marker for distinguishing thyroid hyperplasia from tumors.

To our knowledge, this is the first evaluation of PKA regulatory elements in a relevant series of patients with different pathological conditions, even if more functional



**Fig. 2.** Immunoreactivity scores for PRKAR1A (filled columns) or PRKAR2B (striped columns) in papillary and follicular thyroid carcinomas according to lymph node status (N, **A**) or tumor size (T, **B**). \*,  $p = 0.03$  by Mann-Whitney U test.

studies of PRKAR1A in thyroid cancer are needed to validate its role as a index of malignancy for these neoplasms.

## References

- Calebiro D., de Filippis T., Lucchi S., Martinez F., Porazzi P., Trivellato R., Locati M., Beck-Peccoz P. and Persani L. (2006). Selective modulation of protein kinase A I and II reveals distinct roles in thyroid cell gene expression and growth. *Mol. Endocrinol.* 20, 3196-211.
- Cho-Chung Y.S., Pepe S., Clair T., Budillon A. and Nesterova M. (1995). cAMP-dependent protein kinase: role in normal and malignant growth. *Crit. Rev. Oncol. Hematol.* 21, 33-61.
- Dremier S., Coulonval K., Perpete S., Vandeput F., Fortemaion N., Van Keymeulen A., Deleu S., Ledent C., Clément S., Schurmans S., Dumont J.E., Lamy F., Roger P.P. and Maenhaut C. (2002). The role of cyclic AMP and its effect on protein kinase A in the mitogenic action of thyrotropin on the thyroid cell. *Ann. N.Y. Acad. Sci.* 968, 106-121.
- Feliciello A., Gallo A., Mele E., Porcellini A., Troncone G., Garbi C., Gottesman M.E. and Avvedimento E.V. (2000). The localization and activity of cAMP-dependent protein kinase affect cell cycle progression in thyroid cells. *J. Biol. Chem.* 275, 303-311.
- Griffin K.J., Kirschner L.S., Matyakhina L., Stergiopoulos S.G., Robinson-White A., Lenherr S.M., Weinberg F.D., Claflin E.S., Batista D., Bourdeau I., Voutetakis A., Sandrini F., Meoli E.M., Bauer A.J., Cho-Chung Y.S., Bornstein S.R., Carney J.A. and Stratakis C.A. (2004). A transgenic mouse bearing an antisense construct of regulatory subunit type 1A of protein kinase A develops endocrine and other tumours: comparison with Carney complex and other PRKAR1A induced lesions. *J. Med. Genet.* 41, 923-931.
- Horvath A., Boikos S., Giatzakis C., Robinson-White A., Groussin L., Griffin K.J., Stein E., Levine E., Delimpasi G., Hsiao H.P., Keil M., Heyerdahl S., Matyakhina L., Libè R., Fratticci A., Kirschner L.S., Cramer K., Gaillard R.C., Bertagna X., Carney J.A., Bertherat J., Bossis I. and Stratakis C.A. (2006). Genome-wide scan identifies mutations in the gene encoding phosphodiesterase 11A4 (PDE11A) in individuals with adrenocortical hyperplasia. *Nat. Genet.* 38, 794-800.
- Horvath A., Korde L., Greene M.H., Libe R., Osorio P., Faucz F.R., Raffin-Sanson M.L., Tsang K.M., Drori-Herishanu L., Patronas Y., Remmers E.F., Nikita M.E., Moran J., Greene J., Nesterova M., Merino M., Bertherat J. and Stratakis C.A. (2009). Functional phosphodiesterase 11A mutations may modify the risk of familial and bilateral testicular germ cell tumors. *Cancer Res.* 69, 5301-5306.
- Horvath A., Bertherat J., Groussin L., Guillaud-Bataille M., Tsang K., Cazabat L., Libè R., Remmers E., René-Corail F., Faucz F.R., Clauser E., Calender A., Bertagna X., Carney J.A. and Stratakis C.A. (2010a). Mutations and polymorphisms in the gene encoding regulatory subunit type 1-alpha of protein kinase A (PRKAR1A): an update. *Hum. Mutat.* 31, 369-379.
- Horvath A., Faucz F., Finkelstein G.P., Nikita M.E., Rothenbuhler A., Almeida M., Mericq V. and Stratakis C.A. (2010b). Haplotype analysis of the promoter region of phosphodiesterase type 8B (PDE8B) in correlation with inactivating PDE8B mutation and the serum thyroid-stimulating hormone levels. *Thyroid* 20, 363-7.
- Kirschner L.S., Carney J.A., Pack S.D., Taymans S.E., Giatzakis C., Cho Y.S., Cho-Chung Y.S. and Stratakis C.A. (2000). Mutations of the gene encoding the protein kinase A type I-alpha regulatory subunit in patients with the Carney complex. *Nat. Genet.* 26, 89-92.
- Lania A., Mantovani G., Ferrero S., Pellegrini C., Bondioni S., Peverelli E., Braidotti P., Locatelli M., Zavanone M., Ferrante E., Bosari S., Beck-Peccoz P. and Spada A. (2004). Proliferation of transformed somatotroph cells related to low or absent expression of PKA regulatory subunit 1A protein. *Cancer Res.* 64, 9193-9198.
- Mantovani G., Bondioni S., Alberti L., Gilardini L., Invitti C., Corbetta S., Zappa M.A., Ferrero S., Lania A.G., Bosari S., Beck-Peccoz P. and Spada A. (2009). Protein kinase A regulatory subunits in human adipose tissue: decreased R2B expression and activity in adipocytes from obese subjects. *Diabetes* 58, 620-6.
- Moretti F., Nanni S. and Pontecorvi A. (2000). Molecular pathogenesis of thyroid nodules and cancer. *Baillieres Best. Pract. Res. Clin. Endocrinol. Metab.* 14, 517-539.
- Porcellini A., Messina S., De Gregorio G., Feliciello A., Carlucci A., Barone M., Picascia A., De Blasi A. and Avvedimento E.V. (2003). The expression of the thyroid-stimulating hormone (TSH) receptor and the cAMP-dependent protein kinase RII  $\beta$  regulatory subunit confers TSH-cAMP-dependent growth to mouse fibroblasts. *J. Biol. Chem.* 278, 40621-40630.
- Pringle D.R., Yin Z., Lee A.A., Manchanda P.K., Yu L., Parlow A.F., Jarjoura D., La Perle K. and Kirschner L.S. (2012). Thyroid specific ablation of the Carney Complex gene, PRKAR1A, results in hyperthyroidism and follicular thyroid cancer. *Endocr. Relat. Cancer* 19, 435-446.
- Sandrini F., Matyakhina L., Sarlis N.J., Kirschner L.S., Farmakidis C., Gimm O. and Stratakis C.A. (2002). Regulatory subunit type I-alpha of protein kinase A (PRKAR1A): a tumor-suppressor gene for sporadic thyroid cancer. *Genes Chromosomes Cancer* 35, 182-192.