Histol Histopathol (2007) 22: 409-416

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

H-*RAS* gene expression in human multinodular goiter

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Summary. The *RAS* protooncogene has an important, although not yet established role in thyroid neoplasia. In this study, we evaluated the H-*RAS* mRNA and protein levels in human samples of nontoxic and toxic multinodular goiter samples, according to serum TSH levels. The mean of H-*RAS* mRNA levels in nodules of nontoxic nodular goiter were significantly increased compared to nonnodular tissue (1.49±1.21 vs. 0.94±0.81 AU, P=0.016). Nine of the 18 specimens (50%) of nontoxic multinodular goiter exhibited increased levels of H-*RAS* mRNA. The increased H-*RAS* mRNA levels were paralleled by inRAcreased H-Ras protein levels in about 90% of the cases. Interestingly, no differences were observed in H-*RAS* expression between nodules and adjacent nonnodular tissue in toxic nodular goiters (0.58±0.27 vs. 0.58±0.20 AU, P=0.88). None of the 10 samples from toxic multinodular goiters exhibited overexpression of H-*RAS*. The H-*RAS* expression was positively correlated with thyroglobulin expression (r^2=0.51; P=0.04). In conclusion, we demonstrated increased levels of H-*RAS* mRNA and protein in samples of nontoxic multinodular goiter, indicating that it might be involved in goiter pathogenesis. In contrast, H-*RAS* overexpression was not detected in any of the samples of toxic multinodular goiter, suggesting different mechanisms for cell proliferation in nodular goiter according to thyroid status.

Key words: H-*RAS*, Multinodular goiter, Human, Gene expression, Thyrotoxicosis

Introduction

Cell proliferation is stimulated by extracellular growth factors that activate specific signaling cascades and dictate the orderly sequence of events needed for DNA synthesis and cell division (Fagin, 1998). The *RAS* protooncogenes (H-, K-, N-*RAS*) are members of the superfamily of GTP-binding proteins (Barbacid, 1987). These proteins, wich are membrane-associated and bound to GDP when inactive, play an important role as molecular switches in the transduction of mitogenic signals from growth factor receptors on the cell surface. Activation of *RAS* oncogenes by point mutations in codons 12, 13 and 61, is an early event in thyroid tumorigenesis (Lemoine et al., 1989; Namba et al., 1990; Karga et al., 1991). In contrast to other human tumor types whereby mutations show specificity for one *RAS* gene, mutations of all three genes have been reported in thyroid neoplasias (Lemoine et al., 1989; Suarez et al., 1990; Suarez, 1998). Aberrant expression of the *RAS* genes has been well established in several human cancers, including breast cancer, head and neck cancer and leukemia (Kiaris et al., 1995; Gougopoulou et al., 1996; Miyakis et al., 1998).

Multinodular colloid goiter is characterized by heterogeneity in growth and function of thyroid follicular cells (Derwahl and Studer, 2001). The ultimate causes of multinodular goiter are to be sought within the thyrocytes themselves, in analogy to most other benign neoplasia (Krohn et al., 2005). Intrinsic metabolic and functional differences between the individual thyrocytes, from which new follicles are generated during goitrogenesis, are the cause of the functional and structural heterogeneity (Derwahl and Studer, 2000; Krohn et al., 2005). Studies have demonstrated the predominant neoplastic character of nodular structures and monoclonal origin for the majority of the nodules (60-70%), with somatic mutations as the starting point (Krohn et al., 2005). Furthermore, recent developments in this field also indicate that normal and goitrous thyroids are composed of clustered angiofollicular units controlled by vasoactive and angiogenic factors (Gerard et al., 2000, 2002).

Previous studies have shown that H-*RAS* activation induces proliferation in normal human thyroid epithelial cells without loss of differentiation, as assessed by the expression of thyroid specific genes (Gire and Wynford-Thomas, 2000). In this context, we hypothesized that increased H-*RAS* expression could also play a role in

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human nodular goiter, since this pathology maintains most of the differentiated functions despite the abnormal proliferative rate. Therefore, the present study was designed to evaluate H-*RAS* expression in nodular goiter and determine whether the human disease displays the same findings observed in *in vitro* studies.

Materials and methods

Patients

The study was carried out between February 2000 and August 2002. Consecutive unselected patients with a diagnosis of multinodular goiter attending the Endocrine or Head and Neck Surgery Divisions at Hospital de Clínicas de Porto Alegre were eligible. Multinodular goiter was defined by the presence of two or more nodules detected by physical examination. Surgery was indicated independently by attending physicians and tissues obtained at surgery were immediately frozen until analysis. Tissue samples were analyzed independently by a pathologist, and classified histologically as multinodular goiter, according to WHO recommendations (Hedinger et al., 1988). On functional grounds, the cases were then subclassified as nontoxic or toxic based on serum TSH levels (see below). Thirty-five samples of multinodular goiter were collected. Of them, 7 were excluded because only one nodule was detected by ultrasonography or macroscopic exam. Thus, 28 samples of multinodular goiter were available for the study.

Clinical data were retrospectively evaluated and included medical history, physical examination, and complementary studies in accordance to clinical indication. Serum TSH levels were measured by a double antibody–sensitive assay (Immulite, Diagnostic Products, Llanberis, United Kingdom). The goiter was classified as nontoxic multinodular goiter when the serum TSH value was normal and as toxic multinodular goiter in cases with suppressed TSH values (reference levels: 0.4-4.0 mU/L). Other causes of thyrotoxicosis (Graves' disease, thyroid hormone administration) were excluded in patients with suppressed levels of TSH. The information obtained from the study did not influence or affect the patients' diagnosis or treatment. The Ethics Committee at the Hospital approved the study protocol, and all patients gave their informed consent.

Tissue specimens and RNA preparation

Thyroid samples were obtained from both nodule and adjacent nonnodular tissue at the time of surgery. Samples were collected from the dominant nodule in the nodular gland evaluated by macroscopic examination. Total RNA was isolated from 50-100 mg of thyroid tumor and surrounding nontumor tissues using TRIzol® reagent (Invitrogen™ Life Technologies Inc., NY, USA) according to the manufacturer's instructions. The purity of the total RNA was assessed by UV spectrophotometry (GeneQuant II®, Amersham Pharmacia Biotech).

Reverse transcriptase - polymerase chain reaction (RT-PCR)

RT-PCR was performed using the Superscript Preamplification System for First Strand cDNA Synthesis (Invitrogen™ Life Technologies Inc., NY, USA) using 3 µg of total RNA as template. Specific oligonucleotides derived from the coding region of human H-RAS (sense: 5' GACGGAATATAAGCT GGTGGTGG 3' and antisense: 5' TGATCTGCTC CCTGAACTGGTGG 3'), were used to prime target gene, resulting in a predicted 298 bp fragment. A human ß2-microglobulin primer set (5'-ATCCAGCGTAC TCCAAAGATTCAG-3' and 5'- AAATTGAAAGT TAACTTATGCACGC-3') that generated a 623 bp product was used as an internal control. $ß_2$-microglobulin was co-amplified within the same reaction in order to evaluate inter-sample variation in cDNA contents and PCR efficiency. A preliminary series of PCRs reactions were carried out to determine the range of cDNA concentration and the number of cycles over which the samples should be examined before reaching a plateau (data not shown). The PCR reactions included 2 µl of RT products, and were carried out with Taq DNA polymerase (Invitrogen™ Life Technologies Inc., NY, USA) in a final 50 µl volume. The amplification profile was an initial denaturation step at 94ºC for 3 min, followed by 94ºC for 1 min, annealing at 62ºC and extension at 72ºC for 2 min. Twenty amplification cycles were used, with a final additional extension step at 72ºC for 5 min. The $ß_2$-microglobulin primers were included after the 5th cycle. RT-PCR reactions without cDNA samples were carried out as negative controls. All reactions were performed in duplicate. After amplification, 10µl of the PCR products were analyzed on a 1.5% ethidium bromide agarose gel and the intensity of each band was determined by optic densitometry (arbitrary units, AU) (ImageMaster® VDS, Amersham Pharmacia Biotech). DNA band intensity was normalized against the corresponding values of the $ß_2$-microglobulin band intensity. The mRNA levels for H-*RAS* gene were expressed as the ratio of the intensity of the band in nodule versus the adjacent nonnodular tissue. We considered as overexpression, expression levels higher than 1.5 fold in nodules specimens compared to corresponding normal (Miyakis et al., 1998).

The same procedure described above was used to analyze the thyroglobulin expression in nodule and normal thyroid tissue. Specific oligonucleotides derived from the coding region of human thyroglobulin gene (sense: 5' GACGGAATATAAGCTGGTGGTGG 3' and antisense: 5' TGATCTGCTCCCTGAACTGGTGG 3') were used to prime target cDNA, resulting in a 357 bp product. The cycling conditions used were 94ºC for 3 min, 23 cycles of 94ºC for 1 min, 62ºC for 1 min, 72ºC for 1 min and a final 5 min extension period.

Imunohistochemical analysis

Immunohistochemistry was performed on paraffin sections by an indirect 3-stage immunoenzymatic method (Kimura et al., 1999). Briefly, 4 µm sections were placed on 0.1% poly-lysine-coated slides (Sigma, St. Louis, MO). The antigen retrieval of tissue sections was performed by placing slides in 10 mM sodium citrate buffer (pH 6.0) in a microwave oven set at 600W for 3 consecutive cycles of 4 minutes. Subsequently, the endogenous peroxidase activity was blocked by 3% hydrogen peroxide (H_2O_2). Tissue sections were then incubated overnight with 4 µg/ml mouse monoclonal primary antibody to H-Ras (Santa Cruz Biotechnology, Santa Cruz, CA) in phosphate buffered saline (pH 7.4) with 0.05% Bovine Serum Albumin (BSA) and Tris Buffered Saline (TBS). Subsequently, the sections were incubated with biotinylated secondary antibody (Amersham Pharmacia Biotech, Piscataway NJ) for 2 hours, followed by incubation with extr-Avidin® peroxidase conjugate (Sigma, St. Louis MO). The peroxidase activity was visualized by reaction in 3,3'-diaminobenzidine substrate solution (Sigma, St. Louis MO) containing hydrogen peroxide (H_2O_2). The slides were then washed in tap water overnight, counterstained with Mayer's hematoxilin and mounted with E-Z Mount® (Shandon, Pittsburgh PA). The negative control was performed using mouse monoclonal antibody (IgG1, MOPC-21, Sigma, St. Louis, MO). The positivity of the immunostaining reaction was observed by brown staining under light microscope (Eclipse E600 Nikon, Japan). The H-Ras isoform immunostaining was assessed and scored using the semi-quantitative scale for intensity: negative (-), weak (+), moderate (++), and strong (+++). Researchers (LM, ETK) who were unaware of the results of the H-*RAS* mRNA levels performed the imunohistochemical analysis.

Detection of RAS mutations

Tumor cDNA was evaluated for point mutations at codons 12, 13, and 61 of H-*RAS* by Single-Strand Conformational Polymorphism (SSCP) and by Restriction Fragment Length Variance (RFLV). SSCP was performed as previously described (Hongyo et al., 1993; Garcia-Rostan et al., 2003). Briefly, PCR fragments were denatured in formamide and cooled on ice before loading onto the gel. Separation was carried out in a vertical electrophoresis apparatus in a 12% polyacrylamide-0.8% bis-acrylamide gel at 12ºC, at 60-120 mV for 4-6h. DNA bands were visualized by silver staining according to standard procedures.

Restriction Fragment Length Variance: Mutational analysis of codons 12,13, and 61 on the H-*RAS* genes were performed as previously described (Miyakis et al., 1998). Briefly, 10 µl of the PCR products were digested overnight with the restriction endonuclease *MspI* under conditions recommended by the suppliers. The digested products to which glycerol was added were electrophoresed through a 12% polyacrylamide gel and silver stained.

DNA sequencing - Whenever necessary, the absence of the mutation was confirmed by direct sequencing of the PCR product with an automated sequencer (ABI PRISMTM 310, Applied Biosystems, Foster City, CA) using the Bigdye terminator Kit according to the manufacturer's standard protocol.

Statistical analysis

The results are expressed as median (minimum and maximum) or mean ± SD. Clinical and laboratory characteristics between groups were compared using the Student's t-test or Mann–Whitney's U-test for quantitative variables. To compare the expression levels of genes of interest in nodular with surrounding tissue, paired Student's test was used. The correlation between H-RAS and thyroglobulin expression was assessed by Pearson's rank. $P<0.05$ was considered statistically significant. The Statistical Package for Social Science 13.0 professional software (SPSS, Chicago, IL) was used for statistical analysis.

Results

Patients

The characteristics of the 28 patients with multinodular goiter included in this study are shown in Table 1. Based on serum TSH levels, 18 samples were classified as nontoxic multinodular goiter and 10 as toxic multinodular goiter. There were no significant differences between the two groups with respect to sex distribution (P=1.00) or age (51.6±12.8 vs. 57.4±6.13, P=0.11). However, the mean size of the dominant nodule was significantly higher in patients with toxic multinodular goiter than that in nontoxic goiter (5.06±2.5 vs. 3.26±1.4, P=0.04).

H-RAS expression in nontoxic vs. toxic multinodular goiter

The mRNA levels for H-*RAS* gene are expressed as the ratio of the expression in each nodule versus the expression of corresponding normal tissue. The H-*RAS* mRNA levels in nodules of nontoxic multinodular goiter were higher than those in adjacent nonnodular tissue (1.49±1.21vs. 0.94 ±0.81 AU, P=0.016; Figure 1A).

In contrast, no differences in H-*RAS* expression were observed between nodules and adjacent nonnodular tissue of toxic multinodular goiters (0.58±0.27 vs. 0.58 ±0.20 AU, P=0.88; Fig. 1B). The differential expression in H-*RAS* genes according to thyroid status was further stressed when analyzed in terms of percentage of overexpression. We considered levels higher than 1.5-fold in nodule specimens compared to adjacent nonnodular tissue as overexpression (Miyakis et al., 1998). While 50% (9/18) of the samples from patients

Table 1. Clinical and laboratory features of patients with multinodular goiter, and corresponding H-*RAS* expression levels in dominant nodule.

Case	Sex	Age (yr)	Dominant nodule size (cm)[1]	TSH[2] (mUI/L)	H-*RAS* mRNA nodule/tissue ratio[3]	H-Ras protein
Nontoxic multinodular goiter						
1	F	34	2.9	0.84	3.76*	NE
2	F	63	2.5	1.40	1.62*	+
3	F	47	3.0	0.72	1.70*	-
4	F	39	6.3	1.30	1.22	_
5	F	51	1.7	1.20	0.86	NE
6	F	70	5.0	2.20	2.10*	++
7	F	57	3.0	1.32	5.52*	+++
8	F	60	5.7	0.66	1.72*	+++
9	F	48	1.3	2.40	2.26*	++
10	F	61	NE	2.89	0.71	-
11	F	46	2.0	0.49	1.00	NE
12	F	65	2.5	0.81	0.86	+
13	F	52	2.2	1.58	1.70*	NE
14	F	50	4.0	1.51	1.30	-
15	F	48	2.5	0.86	1.58*	++
16	M	24	3.0	1.10	1.17	_
17	F	74	4.5	0.62	1.37	NE
18	F	39	NE	0.80	1.07	-
Mean ± SD		51.5±12.9	3.26±1.4	1.3±0.7	1.75±1.2	
Toxic multinodular goiter						
19	F	55	7.0	0.08	0.56	NE
20	F	72	-	0.002	0.98	-
21	F	51	5.9	0.09	1.05	NE
22	F	57	5.5	0.03	0.62	+
23	F	54	9.0	0.19	0.88	NE
24	M	58	-	0.2	1.16	NE
25	F	63	2.2	0.31	1.25	NE
26	F	57	3.5	0.33	1.23	NE
27	F	55	1.4	0.3	0.88	NE
28	F	52	6.0	0.28	1.12	NE
Mean ± SD		57.4± 6.1	5.1±2.5	0.16±0.1307	0.97±0.23	

[1]: Evaluated by the pathologist, by macroscopic examination. [2]: TSH reference: 0.4-4.0 um/L. [3]: The expression levels were expressed as the ratio of the intensity of the band in tumor versus corresponding normal tissue. *: Considered as overexpression. NE, not evaluated.

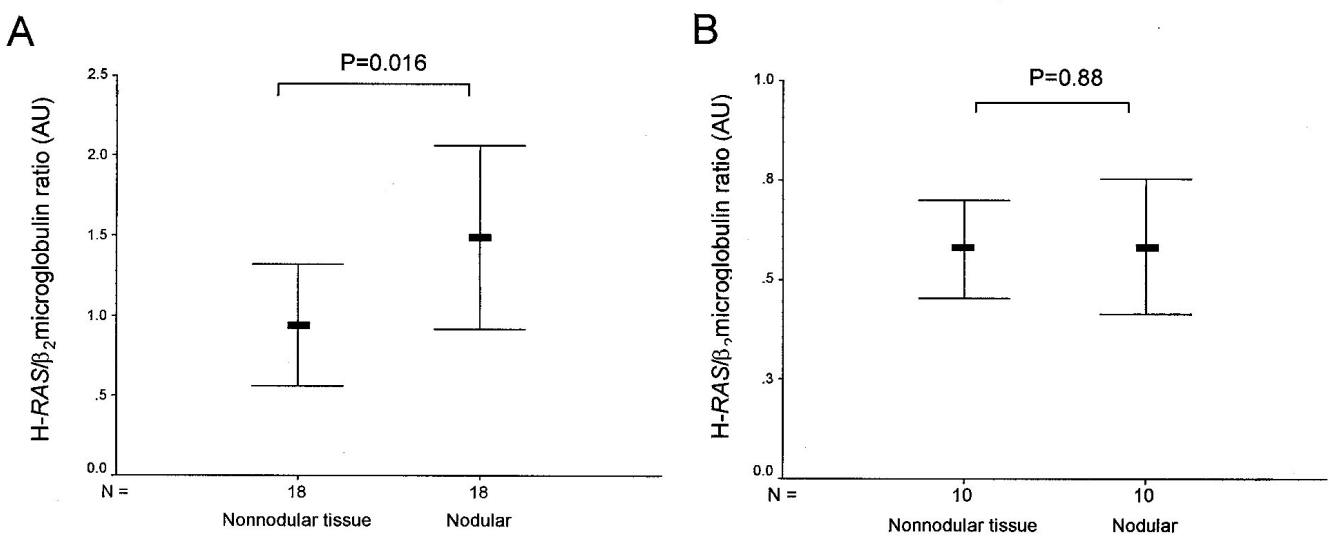

Fig. 1. H-*RAS* mRNA levels in nodules and corresponding nonnodular tissue (arbitrary units). **A.** Nontoxic multinodular goiter. **B.** Toxic nodular goiter. Bars represent mean ± 2 SE.

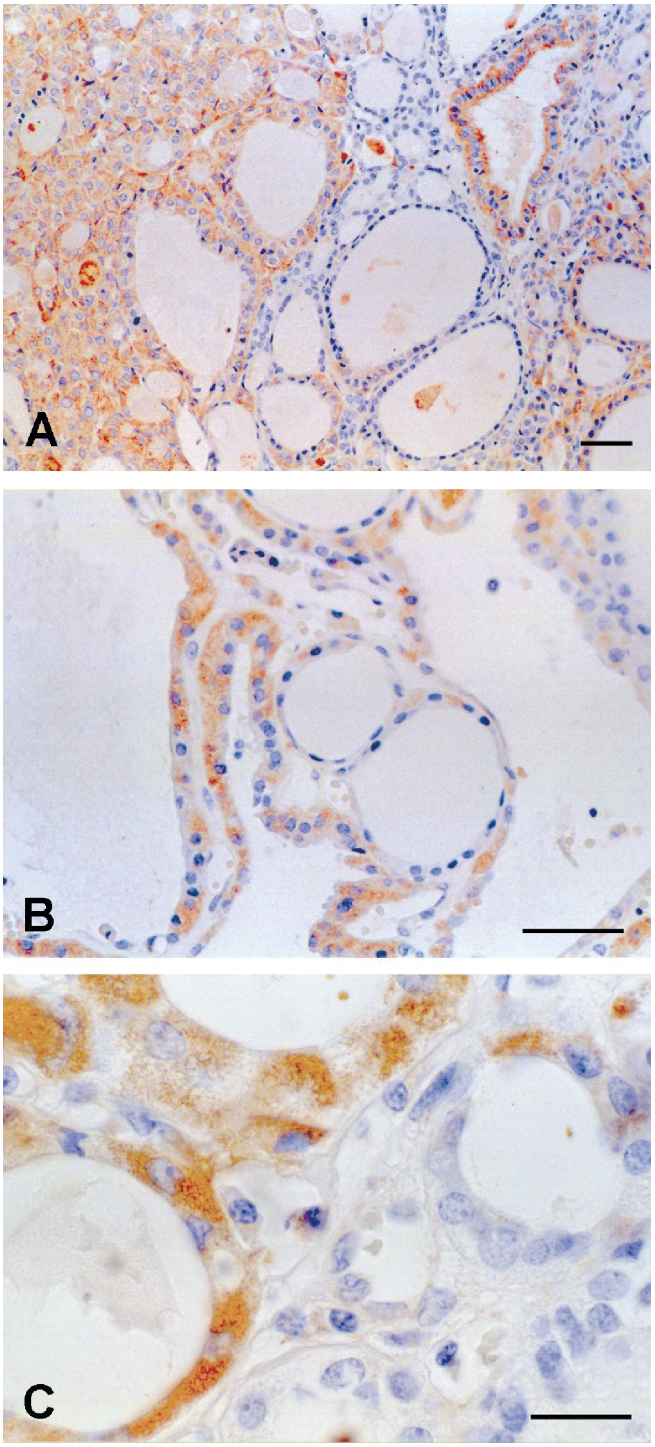

Fig. 2. H-Ras immunostaining in human multinodular goiter sample by the peroxidase method. H-Ras staining is shown in brown and the nuclei are counterstained by Gill's hematoxilin. H-Ras positivity is present in the cytoplasm of both columnar and cuboidal follicular cells while the flat cell follicles are negatives (**A** and **B**, bar: 50 µm). The heterogeneous pattern is also present among neighboring follicles (**C**, bar: 20 µm).

with nontoxic nodular goiter showed overexpression of H-*RAS*, none of the 10 samples from patients with toxic nodular goiter presented overexpression of this protooncogene (Table 1).

We further analyzed the H-Ras protein by immunohistochemical technique. Fifteen cases were available for analysis: 7 with and 8 without H-*RAS* mRNA overexpression. Of the 7 cases with H-*RAS* overexpression, 6 were positive (85.7%) for H-Ras protein. Five of these cases were considered to be of moderate to strong reactivity and 4 have a wide distribution on the tissue (Table 1, Figure 2). Of the 8 cases without H-*RAS* mRNA overexpression, 2 (25%) displayed weak positivity for H-Ras protein (Table1).

To exclude the presence of point mutations, the amplified cDNA fragments including codons 12, 13, and 61 of for H-*RAS* gene were analyzed by PCR-SSCP and RFLV. None of the 24 samples analyzed for H-*RAS* showed mutations. DNA sequencing was performed to confirm the absence of mutations, whenever necessary.

H-RAS vs. thyroglobulin expression in multinodular goiter

Since *in vitro* studies have shown that thyroid cells expressing mutant H-*RAS* retain expression of thyroid-specific proteins, we evaluated whether endogenous human H-*RAS* overexpression was also associated with *in vivo* thyroglobulin expression. Co-amplification of thyroglobulin and H-*RAS* genes were available in 16 out of 23 specimens. Correlation analysis showed that change in H-*RAS* expression was positively and significantly correlated with changes in thyroglobulin expression (r2=0.51, P=0.04; Fig.3).

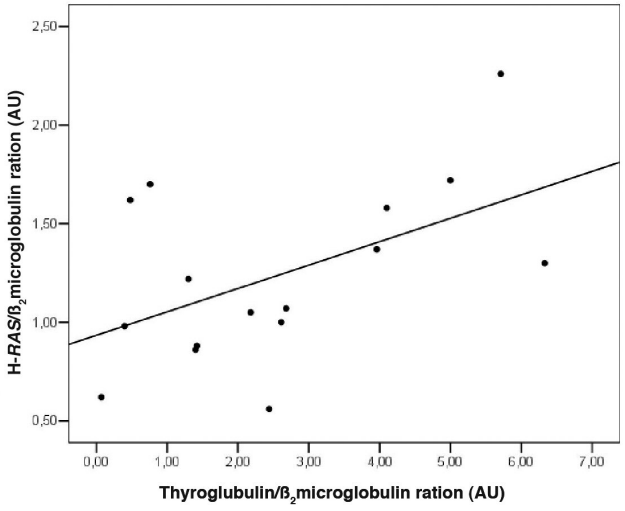

Fig. 3. Correlation of H-*RAS* and thyroglobulin mRNA levels (arbitrary units).

Discussion

The *RAS* protooncogene has an important, not yet established role in the pathogenesis of thyroid neoplasia. In this study, we analyzed the H-*RAS* mRNA and H-Ras protein levels in human multinodular goiter fragments. We observed an increase of nonmutated H-*RAS* mRNA levels in about 50% of dominant nodule samples in nontoxic multinodular goiter. Interestingly, however, none of the samples from patients with suppressed levels of serum TSH showed H-*RAS* overexpression. The H-*RAS* expression was positively and significantly correlated with thyroglobulin expression. These results suggest that an overexpression of normal Ras protein may be implicated in the pathogenesis of nontoxic multinodular goiter.

Ras functions as a molecular switch in a large network of signaling pathways, controlling the differentiation and proliferation of cells. Mutations in *RAS* protooncogenes are prevalent in benignant and malignant thyroid tumors (Lemoine et al., 1989; Suarez et al., 1990; Fagin, 1998). However, the incidence of *RAS* mutations in thyroid tumors and their frequency in specific histological types varies widely in different series (Bartolone et al., 1998; Nikiforova et al., 2003; Krohn and Paschke; 2004). Differences in environmental factors, such as iodine, radiation exposure, histological tumor classifications, or methodology may explain some of the conflicting results. A recent study performed a pooled analysis of 269 mutations garnered from 39 previous studies and observed that *RAS* mutations were significantly less frequent when analyzed by direct sequencing (Vasko et al., 2003). In contrast to solitary thyroid nodules, which have a more homogeneous clinical and pathological picture, nontoxic and toxic multinodular goiters are a miscellaneous group of nodular disease. As a general rule, multinodular goiter development occurs in two phases: activation of thyroid epithelial cell proliferation leading to goiter, and a focal increase of thyroid epithelial cell proliferation causing thyroid nodules (Krohn et al., 2005). Constitutive activation of the RAS/RAF/MEK/ERK/MAP pathway has been suggested as a key mechanism during tumor initiation or progression in thyroid follicular cells (Wynford-Thomas, 1997).

Earlier studies have shown that overproduction of even the normal Ras protein is sufficient to confer a transformation potential on cultured cells (Spandidos and Wilkie, 1984; Zachos and Spandidos, 1997; Adjei, 2001). Indeed, our results demonstrated an increase in H-*RAS* mRNA levels in about 50% of the nodules in nontoxic multinodular goiter, a benign thyroid neoplasia that maintains most differentiated thyroid cell functions. Interestingly, none of the samples from isolated thyroid nodule presented H-*RAS* overexpression (7 samples, data not shown). No statistical differences on morphological or clinical (age, sex, size, range of disease duration, growth rate, and compressive signs) aspects between nodules with or without H-*RAS* overexpression were observed (data not shown). The increased H-*RAS* mRNA levels correlated with higher levels of normal H-Ras protein, which was increased in approximately 90% of nodules with H-*RAS* mRNA overexpression. In agreement with previous studies (Aeschimann et al., 1993), the pattern of immuno-staining was heterogeneous, with coexistence of areas positive and negative for Ras protein in the same gland. No point mutations in codons 12, 13, or 61 of H-*RAS* gene were detected in our samples, in accordance with previous studies that demonstrated a low prevalence of *RAS* mutations in benignant neoplasia (Krohn and Paschke, 2001; Liu et al., 2004). These results allowed us 2 noteworthy inferences: First, cell proliferation in nontoxic multinodular goiter may involve *RAS* signaling. Second, *RAS* overexpression itself does not lead to loss of differentiation in human thyroid follicular cells, as opposed to full transformation observed in rat cell lines (Fusco et al., 1987). Nevertheless, apoptosis seems to be a primary and general response to acute expression of activated Ras in rat thyroid epithelial cells, indicating that Ras might interfere with cell cycle progression and apoptosis (Cheng et al., 2003). Therefore, one could speculate that activation of H-*RAS* in nodules of nontoxic goiter might avoid permanent hyperactivity by promoting thyroid cell apoptosis.

Another interesting finding of this study was the H-*RAS* differential expression between samples from nontoxic and toxic multinodular goiter. We observed that none of the samples from patients with suppressed TSH presented H-*RAS* overexpression. TSH activities are largely mediated by an increase in cAMP intracellular levels, which activate multiple signaling pathways to regulate thyroid differentiation, proliferation, and function. Indeed, constitutive activation of the cAMP signaling pathway is widely accepted as the biochemical driving force of thyroid autonomy, as suggested by the presence of somatic activating TSH receptor mutations in scintigraphically nonsuppressible foci in nontoxic goiters in iodine-deficient areas and TSH receptor mutations and, less frequently, Gs protein mutations in macroscopic toxic thyroid nodules both in solitary nodules and multinodular disease (Krohn and Paschke, 2001). Besides the classical cAMP/protein kinase A (PKA) pathway, cAMP stimulates cell cycle progression through a PKA-independent mechanism that involves the activation of Rap1 and Ras (Rivas and Santisteban, 2003). In rat WRT thyrocytes, overexpression of a constitutive inactive Ras form significantly reduces TSH-mediated proliferation, indicating that Ras is required for the full mitogenic action of TSH (Kupperman et al., 1993). Our results suggest that activation of the TSH-signaling pathway might also be required to induce H-*RAS* expression in human thyrocytes and might indicate different mechanisms for cell proliferation in nodular goiter according to thyroid status. On the other hand, it is also possible that the uncontrolled activation of the cAMP cascade overshadows the H-*RAS* expression. In agreement with

in vitro studies (Gire and Wynford-Thomas, 2000), the H-*RAS* expression levels in human goiter were positively correlated with thyroglobulin expression.

In conclusion, our results demonstrated increased expression of nonmutated H-*RAS* protooncogene in samples of nontoxic multinodular goiter, suggesting that *RAS* genes are involved in the early stages of thyroid cell proliferation and transformation through augmented expression of the normal Ras protein. However, none of the nodule samples from patients with suppressed TSH exhibited increased levels of this oncogene, indicating that distinct mechanisms might explain cell proliferation in nontoxic and toxic multinodular goiter.

Acknowledgements. We thank Drs. Alceu Migliavacca and José Ricardo Guimarães for the thyroid sample biopsies. This study was supported by grants from Conselho Nacional de Desevolvimiento Científico e Tecnológico (CNPq) and Fundo de Incentivo a Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE), Brazil.

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Accepted October 27, 2006