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Specific Pattern of *RAS* Oncogene Mutations in Follicular Thyroid Tumors

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The prevalence of H-*RAS*, K-*RAS*, and N-*RAS* gene mutations in thyroid tumors according to malignancy and histology is controversial. Differences in methodology and histological classifications may explain discrepant results.

To address this issue, we first performed a pooled analysis of 269 mutations garnered from 39 previous studies. Mutations proved significantly less frequent when detected with direct sequencing than without (12.3% *vs.* 17%). The rate of mutation involving N-*RAS* exon 1 (N1) and K-*RAS* exon 2 (K2) was less than 1%. Mutations of codon 61 of N-*RAS* (N2) were significantly more frequent in follicular tumors (19%) than in papillary cancers (5%) and significantly more frequent in malignant (25%) than in benign (14%) tumors. H-*RAS* mutations in codons 12/13 (H1) were found in 2–3% of all types of tumors, but H-*RAS* mutations in codon 61 (H2) were observed in only 1.4% of tumors, and almost all of them were malignant. K-*RAS* mutations in exon 1 were found more often in papillary than follicular cancers (2.7% *vs.* 1.6%) and were sometimes correlated with special epidemiological circumstances.

The second part of this study involved analysis of 80 follicular tumors from patients living in Marseille (France) and Kiev (Ukraine). We used direct sequencing after PCR amplification of exons 1 and 2 of the three *RAS* genes. Common and atypical adenomas were separated using strict cytological criteria. Mutations of H1-*RAS* were found in 12.5% of common adenomas and one follicular carcinoma (2.9%). Mutations of N2-*RAS* occurred in 23.3% and 17.6% of atypical adenomas and follicular carcinomas, respectively. These results confirm the predominance of N2-*RAS* mutations in thyroid follicular tumors and their correlation with malignancy. They support the implication of N2-*RAS* mutations in the malignant progression of thyroid follicular tumors and the assumption that some atypical adenomas are precursors of follicular carcinomas. (*J Clin Endocrinol Metab* 88: 2745–2752, 2003)

BETWEEN 5 AND 10% of individuals develop clinically palpable thyroid nodules in their lifetime (1). In most cases, thyroid nodules correspond to benign follicular neoplasia (adenoma) or hyperplasia. Only 2–5% of them are malignant. According to the World Health Organization (WHO) classification, adenomas can be subdivided into three categories, *i.e.* common, atypical, and Hürthle cell adenomas (2). The main features distinguishing atypical from common adenomas are unusual cellularity, irregular architecture and cytology, and/or presence of numerous mitoses (3). Atypical adenoma resembles carcinoma but without the invasive signs characteristic of follicular carcinoma or the clear overlapping nuclei characteristic of papillary carcinoma (4, 5). However, because these features may be more or less pronounced, even experienced pathologists have trouble making a diagnosis, as shown by the wide interobserver variability for diagnosis of follicular thyroid tumors (6). These problems stem from poor understanding of the early stages of thyroid carcinogenesis and the great heterogeneity of tumors classified as adenoma.

Activating mutations of all three *RAS* oncogenes (H-*RAS*, K-*RAS*, and N-*RAS*) in thyroid tumors were first reported in 1988 (7, 8). Those early results demonstrated that *RAS* mutations were more frequent in follicular than in papillary tumors and that the two types of carcinoma had different mutation patterns (9). The presence of mutations in up to 50% of microfollicular adenomas supported the contention that *RAS* oncogene activation was an early event in follicular thyroid tumorigenesis (10, 11).

In subsequent studies on *RAS* oncogenes in thyroid tumors, various laboratories reported disparate results regarding the incidence of mutations, isoform pattern (H-*RAS*, K-*RAS*, or N-*RAS*), and correlation of mutations with histology. The incidence of *RAS* oncogene mutations ranged from 0–50% in papillary cancer (12–14), 0–85% in adenomas (15, 16), 14–62% in follicular carcinomas (15, 17), and 0–60% in anaplastic carcinomas (10, 18). Although some investigators found no correlation between the mutated *RAS* oncogene isoform and tumor pathology (19), others reported a higher frequency of mutations in the codon 61 of H-*RAS* and N-*RAS* in follicular tumors and poorly differentiated carcinomas (15, 17). Mutations involving K-*RAS* or codons other than codon 61 in H-*RAS* and N-*RAS*, and mutations in other types of thyroid tumors proved uncommon (20, 21).

At least four possible explanations can be offered to account for these disparate findings. The first involves methodology because a wide variety of techniques have been used to detect mutations. In this regard, it should be emphasized that specificity has improved with increasingly wider availability of direct sequencing. Another explanation requiring further study involves environmental factors such as iodine deficiency (16, 22), radiation exposure (23–25), and foodborne carcinogens (14). A third explanation is that the small number of cases in many studies may have had a confounding effect on statistical analysis. The fourth explanation in-

Abbreviations: AFA, Atypical follicular adenoma; FA, follicular adenoma; MIFC, minimally invasive follicular carcinoma; WIFC, widely invasive follicular carcinoma.

volves variations in the histological classification of follicular thyroid tumors, but the impact of such variations is difficult to evaluate because little attention has been given to morphological and biological heterogeneity of thyroid follicular tumors.

The purpose of this article is to describe and compare the results of assessment of *RAS*-oncogene mutations in 80 thyroid follicular tumors in patients from Marseille, France, and Kiev, Ukraine. Tumor diagnosis was based on strict morphological criteria. Mutations were assessed by direct sequencing of PCR-amplified sequences in exons 1 and 2 of the H-*RAS*, K-*RAS*, and N-*RAS* oncogenes. Findings were compared with the results of a pooled analysis of 39 previous reports on *RAS*-oncogene mutations in thyroid tumors, with special attention to the influence of methodology and histological classification.

Patients and Methods

Patients and tissue

Tissue specimens were obtained from 33 patients treated at the Ukrainian Center of Endocrine Surgery in Kiev, Ukraine, and 47 patients treated at the Timone University Hospital Center in Marseille, France. Tumors were classified as widely invasive follicular carcinoma (WIFC) in 16 cases, minimally invasive follicular carcinoma (MIFC) in 18, atypical follicular adenoma (AFA) in 30, and classical follicular adenoma (FA) in 16. The clinicopathological features of these tumors are listed in Table 1. The study protocol was approved by the clinical research committee of Marseille Public Hospital System.

After fixation in 10% formalin and embedding in paraffin, 5 μm- and 20 μm-thick serial sections were made for each specimen. The 5-μm sections were stained with hematoxylin and eosin and examined by two pathologists (C.d.M. and V.V.). Histological typing was based on WHO criteria (2). Follicular adenoma exhibited a regular predominantly microfollicular architecture comprising cells with small, round, equal-sized, regularly spaced normochromatic nuclei. Atypical adenoma had microfollicular, solid, or trabecular architecture comprising epithelial cells with one or more of the following nuclear features: overlapping, uneven size, irregular borders, pale chromatin, and prominent nucleoli. Vascular or capsular invasion was excluded after examination of one inclusion block per 5 mm of tumor. No tumor displayed nuclear features typical of papillary cancer. Follicular carcinoma presented the same general features as atypical adenoma but in association with capsular or vascular invasion. Follicular carcinoma was further subdivided into MIFC and WIFC. Ten patients, including one with MIFC and nine with WIFC, had distant metastasis at the time of diagnosis.

DNA extraction

The unstained 20-μm tumor sections were used for *RAS* oncogene detection as follows. Slides were deparaffinized in xylene, washed in ethanol, and rehydrated. Any tissue surrounding the tumor, including normal thyroid, connective tissue, inflammatory cells, and necrotic or hemorrhagic zones, was carefully pared away using a scalpel under microscopic observation. The purpose of paring was to ensure that tumor cells comprised over 90% of the remaining specimen. After suspension in 400 μl of 100 mM Tris buffer (pH 8.0) containing 100 mM NaCl, 20 mM EDTA, 2% sodium dodecyl sulfate, and 80 μg proteinase K (Roche Diagnosis, Meylan, France), pared specimens were incubated for 5 d at 55 C with daily addition of 80 μg proteinase K. At the end of incubation, proteinase K was inactivated by heating for 10 min at 95 C. DNA was extracted using the QI-Amp DNA mini kit (QIAGEN, Les Ulis, France) according to the manufacturer's tissue protocol. DNA content was quantified by spectrophotometric absorption at 260 nm and evaluation of A 260/A 280 ratio. Extracts were stored at 4 C until assayed.

PCR amplification and sequencing

Sequences of H-*RAS*, K-*RAS*, and N-*RAS* oncogenes in exons 1 and 2 (designated H1, H2, K1, K2, N1, and N2) were amplified using the primer-pairs listed in Table 2. The PCR mixture (50 μl) contained 0.1–0.5 μg of genomic DNA, 2 mM or 1.5 mM $MgCl_2$ for the K1-*RAS* and N1-*RAS* oncogenes or the other *RAS* oncogenes, respectively, 10× concentrated PCR-buffer (QIAGEN), 200 μM of deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 200 nM of each primer, and 1.25 U of HotStar Taq DNA Polymerase (QIAGEN). Amplification was achieved on a Cyclogen Dri-block Cycler Techne (Cambridge Ltd., Cambridge, UK). After HotStarTaq DNA-polymerase activation at 95 C for 15 min, templates were denaturated at 94 C for 2 min. This initial step was followed by 30–38 cycles of PCR, each comprising 1 min of denaturation at 94 C, 1 min of annealing (at 53 C for N2-*RAS*; 55 C for H1-*RAS* and K1-*RAS* oncogene; 57 C for H2-*RAS* and N1-*RAS* oncogene; and 58 C for K2-*RAS* oncogene), and 1 min of extension at 72 C. In the last cycle, the extension step was prolonged for 10 min. PCR products were submitted to electrophoresis on 3% agarose gel in Tris-acetate-EDTA buffer and stained with ethidium bromide. In all cases, direct sequencing was performed using an Applied Biosystem 373XL sequencer (PE Applied Biosystems, Paris, France) according to the manufacturer's instructions on PCR products purified using a QIAGEN gel extraction kit. In samples exhibiting mutations, both sense and antisense strands were sequenced for confirmation.

Cumulated analysis of published reports of RAS *mutations*

A pooled analysis including 39 previously published reports focusing on *RAS* oncogene mutations in thyroid tumors was performed. The purpose of this analysis was to gain better insight into the incidence of *RAS* oncogene mutations and clinicopathological correlations in a large patient population. Selection of reports for inclusion in this pooled analysis was based on the thoroughness of study data regarding methodology, histology, and *RAS* oncogene mutations (8–11, 14–47). The histological tumor type, the method used to detect mutations, and the presence or absence, type, and location of mutations were noted in all

TABLE 2. Primer pairs used to amplify H-*RAS*, K-*RAS*, and N-*RAS* gene sequences

Gene	Codon	Name	Length (bp)	Primer sequences (5′–3′; a, forward; b, reverse)
H-*RAS*	12/13	H1	123	a-ATGACGGAATATAAGCTGGT
				b-CTCTATAGTGGGGTCGTATT
H-*RAS*	61	H2	178	a-AGGTGGTCATTGATGGGGAG
				b-AGGAAGCCCTCCCCGGTGCG
K-*RAS*	12/13	K1	164	a-GGCCTGCTGAAAATGACTGAA
				b-GGTCCTGCACCAGTAATATGC
K-*RAS*	61	K2	133	a-CAGGATTCCTACAGGAAGCAAGTAG
				b-CACAAAGAAAGCCCTCCCCA
N-*RAS*	12/13	N1	112	a-ATGACTGAGTACAAACTGGT
				b-CTCTATGGTGGGATCATATT
N-*RAS*	61	N2	176	a-TCTTACAGAAAACAAGTGGT
				b-GTAGAGGTTAATATCCGCAA

TABLE 1. Clinicopathological findings in 80 thyroid tumors

Diagnosis	No.	Gender (M/F)	Mean age (yr)	Mean size (mm)	Metastasis	Nodule setting (solitary/goiter)
FA	16	3/13	44.3 ± 16.1	22.1 ± 8.9	0	11/5
AFA	30	5/25	45 ± 13.6	25.9 ± 10.2	0	16/14
MIFC	18	6/12	46.3 ± 18.6	33.3 ± 8.2	1	9/9
HIFC	16	5/11	50.5 ± 17.2	42.5 ± 16.5	9	6/10

cases. If mentioned, data on iodine intake, radiation exposure, or other epidemiological factors were also noted.

Statistical analysis

Correlations between clinicopathological variables and *RAS* mutations were analyzed using the χ^2 or Fischer's exact test. A P value less than 0.05 was considered as significant.

Results

RAS mutations in 80 thyroid follicular tumors

Results on *RAS* mutations found in our study are detailed in Table 3. Their correlation with tumor histology is shown in Fig. 1.

Mutations were found only in H1-*RAS* and N2-*RAS*. H1-*RAS* mutation was a single base substitution (GGC→GTC) at codon 12 resulting in an amino acid change from *Gly* to *Val.* It was found in two FA from French patients (12.5%) and one MIFC from a Ukrainian patient (5.5%). Reexamination of the slides of the two FA confirmed the absence of cellular atypia and revealed no special feature as compared with nonmutated tumors. The difference in the incidence of H-*RAS* mutation between the FA and FC subgroups was not statistically significant ($P = 0.1$), indicating that H1-*RAS* mutations occur in benign as well as in malignant follicular tumors.

N2-*RAS* mutations were found in seven AFA (23.3%), four MIFC (22.2%), and two WIFC (12.5%). In one MIFC, it was a CAA→AAA transversion at codon 61 resulting in an amino acid change from *Gln* to *Lys.* In the other 12 cases, CAA→CGA transitions at codon 61 resulting in a *Gln* to *Arg* change were found. Reexamination of these slides confirmed characteristic features of AFA and MIFC differing only with regard to the presence of invasive properties in the MIFC. Growth pattern was heterogeneous, with unevenly distributed compact areas. The appearance of follicular cells was polymorphous, with frequently enlarged eosinophilic or clear cytoplasms. Nuclei were enlarged and often overlapping, but they retained their rounded shape. Nuclear chromatin exhibited a dusty appearance with dispersed clumping creating a salt-and-pepper effect. The difference in the incidence of N2-*RAS* mutation was statistically significant between FA (0%) and AFA (23.3%; $P = 0.038$), as well as between FA (0%) and MIFC (17.6%; $P = 0.026$). Conversely, the difference in the incidence of N2-*RAS* mutation between AFA (23.3%) and FC (17.6%) was not significant ($\chi^2 = 0.37$; $P > 0.5$). The observed difference in the incidence of N2-*RAS* mutation between MIFC (22.2%) and WIFC (12.5%) was not statistically significant ($\chi^2 = 0.56$; $P > 0.3$).

Clinicopathological features of mutated cases are given in Table 4. No correlation was found between the incidence of N2-*RAS* mutation and any of the following parameters: age, sex, tumor size, metastasis, and geographical origin (France or Ukraine). The only pathological parameter significantly correlated with N2-*RAS* mutations in follicular tumors was association with multinodular goiter ($\chi^2 = 5.2$; $P < 0.05$).

TABLE 3. *RAS* mutations in 80 follicular thyroid tumors

Ras gene	FA[a]	AFA[a]	MIFC[a]	WIFC[a]
H1-*RAS*	2/16 (12.5%)	0/30	1/18 (5.5%)	0/16
H2-*RAS*	0/16	0/17	0/8	0/8
K1-*RAS*	0/16	0/17	0/8	0/8
K2-*RAS*	0/16	0/17	0/8	0/8
N1-*RAS*	0/16	0/17	0/8	0/8
N2-*RAS*	0/16	7/30 (23.3%)	4/18 (22.2%)	2/16 (12.5%)

[a] Number of positive cases/total number of studied cases (percentage).

Pooled analysis of published reports on RAS oncogene mutations in thyroid tumors

Because direct sequencing is the only means of definitely proving mutation, we compared the overall incidence of mutations found in all studies (n = 39) and studies including confirmation of mutation by direct sequencing (n = 22; Table 5). We first analyzed a subset of 27 studies in which estimation of the incidence of *RAS* mutation was based on analysis of all three *RAS* oncogene isoforms, *i.e.* H-*RAS*, K-*RAS*, and N-*RAS*, in the same tumors (Table 5; Refs. 8–10, 14–17, 19–25, 27–30, 33, 37, 38, 40, 41, 44, 45, 48, 49). Next, we pooled the

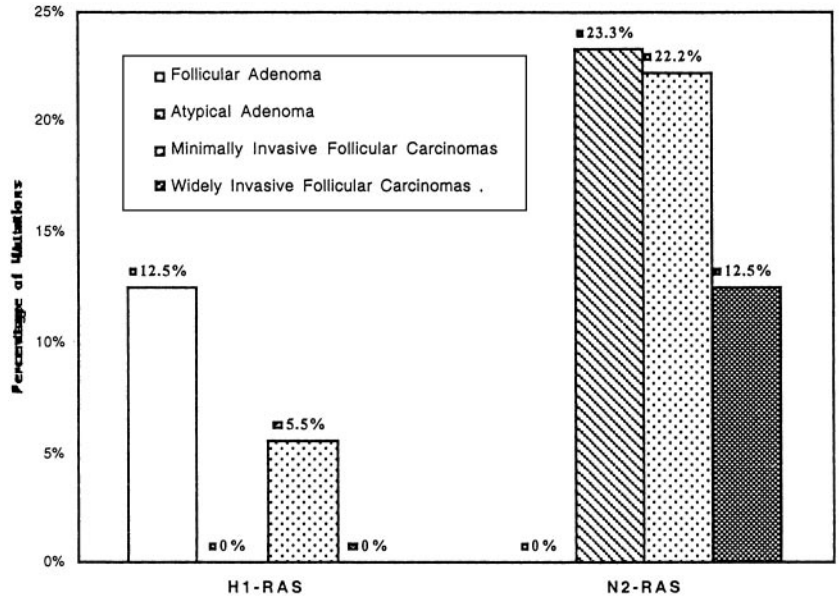

FIG. 1. Frequency of H1-*RAS* and N2-*RAS* mutations found in our study according to the histology of thyroid tumors.

TABLE 4. Clinical features of follicular thyroid tumors displaying *RAS* mutations

Diagnosis	Country	Age (yr)	Gender	Size (mm)	Nodularity	Metastasis	Mutated *RAS*
FA	Fr	37	M	20	S	−	H1
FA	Fr	26	F	15	S	−	H1
AFA	U	37	F	16	S	−	N2
AFA	U	63	F	35	G	−	N2
AFA	U	42	F	20	G	−	N2
AFA	Fr	48	F	25	G	−	N2
AFA	Fr	31	F	30	G	−	N2
AFA	Fr	55	M	25	S	−	N2
AFA	Fr	45	F	20	G	−	N2
MIFC	U	63	F	30	G	−	N2
MIFC	U	37	F	25	G	−	N2
MIFC	Fr	56	F	30	G	−	N2
MIFC	Fr	32	F	25	G	−	N2
WIFC	Fr	32	F	60	G	+	N2
WIFC	Fr	68	F	80	G	+	N2

Fr, France; U, Ukraine; M, male; F, female; S, solitary nodule; G, goiter.

TABLE 5. Incidence of *RAS* mutations in thyroid tumors according to the detection method

Ras oncogenes	Detection method[a]		*P*
	All methods[b]	Mutations confirmed by direct sequencing[c]	
H-, N-, and K-*RAS*[d]			
Exons 1 and 2	171/1003 (17%)*	69/562 (12.3%)	<0.01
H-*RAS*			
Exon 1	60/1434 (4.2%)	25/768 (3.2%)	ns
Exon 2	35/1220 (2.9%)	10/768 (1.3%)	<0.01
K-*RAS*			
Exon 1	43/1564 (2.8%)	18/978 (1.8%)	ns
Exon 2	6/1121 (0.5%)	3/669 (0.4%)	ns
N-*RAS*			
Exon 1	15/1434 (1%)	3/924 (0.3%)	<0.04
Exon 2	110/1377 (8%)	61/751 (8%)	ns

ns, Not significant.

[a] Number of positive cases/total number of cases (percentage).

[b] This column pools results of 39 studies performed using any method to detect *RAS* mutations with or without a direct sequencing step.

[c] This column pools results of 22 studies in which *RAS* mutations detected as above were confirmed by direct sequencing.

[d] The three isoforms H-, N-, and K-*RAS* were analyzed in the same tumors.

results of studies in which any of the H-*RAS*, K-*RAS*, and N-*RAS* isoforms were analyzed to know their respective prevalence (Table 5). The overall rate of mutations was significantly lower when estimated with direct sequencing than without (12.3% *vs.* 17%; $P < 0.01$). Overestimation of H2-*RAS* and N1-*RAS* mutations was particularly frequent without sequencing (1.3% and 0.3% *vs.* 2.9% and 1%, respectively; $P < 0.01$ and $P < 0.05$). The most common mutations in thyroid tumors affected N2-*RAS* in codon 61. The rate of mutation involving N2-*RAS* oncogenes was significantly higher than the rate of mutations involving the H1-*RAS*, H2-*RAS*, and K1-*RAS* oncogenes, *i.e.* 8% *vs.* 3.2%, 1.3%, or 1.8%, respectively ($P < 10^{-6}$). The least frequent mutations involved N1-*RAS* (0.3%) and K2-*RAS* (0.4%).

On the basis of previous findings, we focused analysis of the prevalence of *RAS* mutations according to tumor type on 22 studies in which mutations (n = 120) were confirmed by direct sequencing (14, 17, 18, 20, 21, 24, 25, 28, 30, 32–38, 40, 41, 43, 45, 46). Results are shown in Table 6 and illustrated in Fig. 2. For comparison, results of our study on follicular adenomas and carcinomas have been repeated in Table 6 after those of pooled analysis. For anaplastic carcinomas, subtypes of follicular tumors and subtypes of papillary cancers, the number of cases in which detection of the three isoforms of *RAS* was performed was too small for statistical analysis. Mutations involving H1-*RAS* were found in 2–3% of all tumors, with no significant correlation with any specific class. Mutations involving N1-*RAS* and K2-*RAS* were equally rare in all tumors (0–2%). Mutations involving H2-*RAS* were rare, but they were significantly more frequent in follicular carcinomas than in benign tumors (5% *vs.* 0.5%; $P < 0.02$). Regarding mutations involving K1-*RAS*, the difference in prevalence was not significant between papillary (2.3%) and follicular cancer (1.6%), but it was significantly higher for malignant (papillary and follicular carcinoma) than benign thyroid tumors (colloid nodules and adenoma; $P < 10^{-4}$). Mutations in codon 61 of N-*RAS* accounted for more than 50% of *RAS* mutations observed in thyroid tumors. These mutations were more frequent in follicular tumors than in other histotypes ($P < 10^{-7}$), and their prevalence was significantly higher in carcinoma (25%) than in adenoma (14%; $P < 0.03$). They were found in 5% of papillary cancers, but the histological subtype of these cases was not specified.

We also analyzed the type and localization of the 120 *RAS* mutations confirmed by direct sequencing. Mutations were found in benign tumors in 35 cases and malignant tumors in 85 cases ($P < 10^{-8}$). There were 64 transitions and 56 transversions. Mutations affected exon 1 in 44 cases (37.5%) and exon 2 in 76 cases (62.5%; $P < 0.001$). Transitions were more frequent in exon 2 (n = 51) than in exon 1 (n = 13), whereas transversions were more frequent in exon 1 (n = 33) than in exon 2 (n = 23; $P < 0.0001$). The most frequent mutation in exon 1, *i.e.* GGC→GTC, resulting in *Gly* to *Val* substitution, accounted for 62% of exon 1 mutations. The prevalence of this mutation was the same in all the histological types. In 15.5% of cases, mutations in exon 1 affecting codons 12 (GGT→AGT), 13 (GGC→AGC), or 15 (CCG→TCG) resulted in *Gly* to *Ser* substitution. Interestingly, this type of mutation was found almost exclusively in thyroid papillary or anaplastic carcinomas and sometimes showed geographical con-

TABLE 6. Frequency of *RAS* mutations according to the histology of thyroid tumors in the present study and studies from literature

Histology	H-*RAS*		K-*RAS*		N-*RAS*	
	Exon 1	Exon 2	Exon 1	Exon 2	Exon 1	Exon 2
Colloid nodules						
Pooled literature analysis	3/90 (3%)	0/90	1/90 (1%)	0/81	1/90 (1%)	2/81 (2.5%)
Adenomas						
Pooled literature analysis	2/105 (1.9%)	1/105 (1%)	1/153 (0.6%)	2/111 (1.8%)	0/135	16/114 (14%)
Present study	2/46 (4.3%)	0/33	0/33	0/33	0/33	7/46 (15.2%)
P value	ns	ns	ns	ns	ns	ns
Follicular carcinomas						
Pooled literature analysis	2/84 (2.4%)	4/81 (4.9%)	2/124 (1.6%)	0/77	1/116 (0.9%)	21/88 (23.9%)
Present study	1/34 (2%)	0/16	0/16	0/16	0/16	6/34 (17.7%)
P value	ns	ns	ns	ns	ns	ns
Papillary carcinomas						
Pooled literature analysis	7/345 (2%)	2/345 (0.6%)	11/402 (2.7%)	1/292 (0.3%)	1/378 (0.3%)	18/354 (5%)

ns, Not significant.

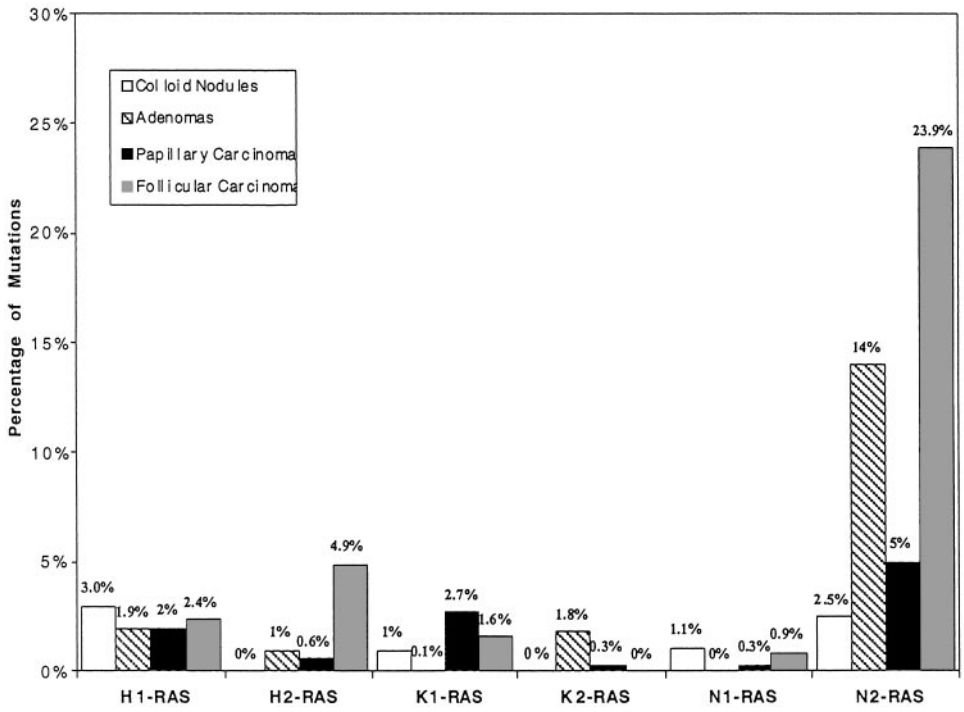

FIG. 2. Pooled analysis: frequency of *RAS* mutations according to the histology of thyroid tumors.

centration (14). Mutations in exon 2 affecting codon 61 resulted in *Gln* to *Arg* changes in 66.6% of cases (CAG→CGG or CAA→CGA) and *Gln* to *Lys* changes in 25.3% of cases (CAG→AAG or CAA→AAA). These mutations involved follicular tumors in 68% of cases, including 67% that were malignant.

Discussion

RAS oncogenes are key components in the regulation of cell growth and differentiation (51). In up to 35% of human tumors, constitutively activated mutant ras oncogenes have been found involving different isoforms (H-*RAS*, K-*RAS*, and N-*RAS*), depending on the tissue type. Numerous studies have demonstrated *RAS* mutations in human thyroid tumors (52), and the ability of activated *RAS* to induce thyroid neoplasms has been demonstrated in both *in vivo* (53, 54) and *in vitro* (55) experiments. However, widely disparate findings have been reported concerning the mutated *RAS* isoforms in different thyroid tumors and the correlation of mutations with histology, epidemiology, and malignancy.

A major limitation for the study of *RAS* oncogene mutations in thyroid tumors has been the small number of cases in individual series. To overcome this problem, we performed a pooled analysis of 229 cases of *RAS* mutations described in 39 previous publications and compared the findings with data from our own study of 80 follicular thyroid tumors. Preliminary findings of pooled analysis indicated that detection of *RAS* mutations was less frequent using direct sequencing than other methods (12.3% *vs.* 17%). This may be due in part to the inability of direct sequencing to detect a low proportion of mutated alleles in polyclonal tumors or samples including a proportion of normal tissue. However, similar results were found in several large studies in which tumors of all histological types were analyzed using a highly sensitive screening method followed by direct sequencing to confirm mutations (20, 21, 25). An alternative

explanation is that some studies using highly sensitive but less specific screening methods without confirming mutations by direct sequencing may have overestimated *RAS* mutations. Because direct sequencing is the gold standard in mutation detection, we chose it in our study to ensure the most reliable results. To avoid dilution of mutated alleles in heterogeneous tissues, we studied homogeneous tumors with well defined histology and pared off normal thyroid tissue from sections under microscopic examination before nucleic acid extraction so that tumor cells represented more than 90% of the sample.

In addition to confirming the low overall incidence of *RAS* mutation in thyroid tumors, the three major findings of our metaanalysis are that *RAS* oncogene mutations are significantly more frequent in malignant than benign tumors, they involve exon 2 (at codon 61) more often than exon 1, and they predominate in follicular tumors over other tumor types. These tumor-specific differences are consistent not only with the general biology of *RAS* oncogenes (51) but also with the initial findings concerning *RAS* oncogene activation in thyroid tumors (9, 56).

The most common mutations in exon 1 are transversions affecting the H-*RAS* and K-*RAS* at an equal rate. Whereas H1-*RAS* mutations show no specific correlation with any tumor type or with malignancy, K1-*RAS* mutations are found mainly in malignant tumors and, in some cases (mutation in codons 12, 13, or 15 leading to a *Gly* to *Ser* change), are strongly correlated with the papillary phenotype. The *Gly* to *Ser* substitution in codons 12, 13, or 15 of *RAS* oncogene could represent a genetic defect implicated in the genesis of some papillary cancers. It is noteworthy that *Gly* to *Ser* changes at position 12 of K-*RAS* oncogene were found in a series of papillary tumors in Thailand but not in Japan. This could indicate involvement of a specific environmental agent (14). Food contamination by N-nitroso compounds has been implicated on the basis of experimental studies showing that nitrosamines preferentially induce mutations in exon 1 of H-*RAS* or K-*RAS* oncogenes (10, 53).

Most mutations in exon 2 are transitions at position 61 involving N-*RAS* oncogenes. The frequency of these mutations is significantly correlated with follicular phenotype and malignancy, but they are also found in some adenomas. The presence of *RAS* mutations in benign thyroid tumors and the observation of self-limiting proliferation after transfection of human thyroid cells with mutant *RAS* (55) have been considered as evidence that *RAS* mutation is unrelated to malignant transformation (57). However, this interpretation is inconsistent with the observation of a higher rate of mutation in malignant than benign thyroid tumors. Thus, the significance of *RAS* oncogene mutation in benign thyroid tumors can still only be speculated upon, especially in the absence of data concerning histological subtypes. Problems in the histological diagnosis of follicular thyroid tumors, *e.g.* interlaboratory variability, have further confused the situation.

The purpose of our study was to evaluate the occurrence of *RAS* mutations in morphologically well defined benign and malignant follicular tumors. We found no mutations involving H2-*RAS*, K1- *RAS*, K2-*RAS*, or N1-*RAS*. These findings are consistent with the conclusion of pooled analysis that such mutations are uncommon in follicular tumors. Observation of mutations involving H1-*RAS* in only two adenomas and one carcinoma is also in agreement with literature analysis showing a low overall incidence (2–3%) of H1-*RAS* mutations in thyroid tumors without significant correlation with histology or malignancy. These findings are also consistent with experimental results showing that transfection of human thyroid follicular cells with mutant (Val-12) H-*RAS* oncogene induces proliferation without transformation or loss of differentiation (55). This body of evidence demonstrates that mutant (Val-12) H-*RAS* oncogene does not cause malignant transformation, although it may be involved in some events initiating thyroid tumorigenesis.

Our data also corroborate results of previous studies showing that N-*RAS* mutation in codon 61 is the predominant *RAS* mutation in follicular thyroid tumors (9, 20). In addition, our data show that N2-*RAS* mutations occur only in carcinomas and atypical adenomas. Nonatypical benign follicular tumors never exhibited these mutations. Because histological subtypes of thyroid adenomas have rarely been analyzed separately in previous studies, there is little information on the prevalence of N2-*RAS* mutations in atypical adenomas. One study comprising a total of 17 atypical adenomas found 5 mutations in codon 12 of H-*RAS* but made no mention of codon 61 or of tumor histology. Because the same study demonstrated several cases of Hürthle cell carcinomas associated with H-*RAS* mutations, the possibility that the atypical adenomas were also of the Hürthle-cell type cannot be ruled out. A separate analysis will be necessary to settle this issue because Hürthle-cell tumors represent a special phenotype (18, 36). Our series did not include any case of Hürthle-cell tumor. To our knowledge, N2-*RAS* mutations have been reported in only two cases of atypical adenomas defined on the basis of strict morphological criteria like those used in our study (35). In this regard, it should be emphasized that the significance of cellular atypia in follicular tumors remains controversial. Although they are regarded as benign tumors, several properties suggestive of malignancy, such as DNA copy depletion (58), loss of heterozygosity (59), alteration of thyroid-peroxidase expression (60, 61), increased proliferation (62), or spectral pattern by proton magnetic resonance (22, 63), are found in atypical adenomas as well as in carcinomas. Evidence that the incidence and type of N2-*RAS* mutations is similar in carcinomas and atypical adenomas further supports the hypothesis that such mutations are implicated in malignant progression and that atypical adenomas have started that progression: they might represent the preinvasive form of follicular thyroid carcinoma.

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