

ras Mutations Are Associated With Aggressive Tumor Phenotypes and Poor Prognosis in Thyroid Cancer

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Purpose: *ras* oncogenic activation has long been demonstrated in thyroid carcinomas of follicular cell derivation, but no consistent relationship has been shown between mutations and clinicopathologic features.

Materials and Methods: We analyzed H-, K-, and N-*ras* mutations by polymerase chain reaction–single-strand conformational polymorphism followed by DNA sequencing in 125 thyroid carcinoma specimens from 107 patients, to include tumors covering the entire spectrum of thyroid tumor differentiation.

Results: Mutations were identified in four (8.2%) of 49 well-differentiated carcinomas (WDCs; two [6.7%] of 30 of the tumors were papillary carcinomas, two [10.5%] of 19 of them were follicular carcinomas), in 16 (55.2%) of 29 poorly differentiated carcinomas (PDCs), and in 15 (51.7%) of 29 undifferentiated carcinomas, with a significant association between *ras* mutation and poorly or undifferentiated tumors ($P < .001$). Twenty-six (74.3%) of 35 patients with

ras-mutated tumors died as a result of disease as opposed to 23 (31.9%) of 72 patients with tumors lacking the mutations. Among patients with differentiated thyroid carcinomas (WDC and PDC), 11 (55.0%) of 20 patients with mutated tumors died as a result of disease as opposed to nine (15.5%) of 58 patients with wild-type *ras* tumors, and the correlation was independent of tumor differentiation and stage ($P = .016$). K-*ras* codon 13 mutations (all with G-A nucleotide transitions resulting in Gly>Asp substitution) and single activating mutations in any of the *ras* genes were also independent predictors of poor survival in differentiated thyroid carcinomas ($P = .027$ and $P = .007$, respectively).

Conclusion: These findings demonstrate that *ras* mutations are a marker for aggressive cancer behavior and indicate a possible role of *ras* genotyping to identify thyroid carcinoma subsets associated with poor prognosis.

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THE THREE members of the *ras* gene family (H-, K-, and N-*ras*) encode membrane-associated guanine nucleotide-binding proteins (p21^{ras}). Point mutations affecting the guanosine triphosphate (GTP)-binding domain (codons 12/13) or the GTPase domain (codon 61) determine the replacement of specific amino acid residues that lock p21^{ras} in the active GTP-bound form, resulting in constitutive activation of the protein and tumor development.¹ Oncogenic mutations of H-, K-, and N-*ras* were among the first genetic changes to be identified in tumors originating from the thyroid follicular epithelium, and numerous reports have documented their occurrence in many different types of thyroid tumors.¹ The

prevalence of *ras* mutations shows considerable variability among the different series, and environmental factors such as radiation exposure may influence both occurrence and pattern of *ras* mutation.²⁻⁴

Despite the numerous reports, relatively few studies have analyzed the significance of the *ras* mutation status for tumor prognosis and its impact on survival. The few studies that have fully addressed these issues have been limited to specific types of thyroid cancer or of *ras* mutation.⁵ To analyze the influence of oncogenic *ras* on the patient's clinical course, we have therefore genotyped for H-, K-, and N-*ras* more than 100 thyroid carcinomas to include the entire spectrum of differentiation from well-differentiated carcinomas (WDCs) to undifferentiated (anaplastic) carcinomas (UDCs). Polymerase chain reaction–single-strand conformational polymorphism (PCR-SSCP), followed by DNA sequencing of each individual shifted band was used for mutation detection.

MATERIALS AND METHODS

Patients With Tumors

We analyzed 107 patients who underwent surgery for thyroid carcinoma. Diagnostic material on these patients was retrieved from the files of the Pathology Departments at Yale New Haven Hospital, Yale University (New Haven, CT; 55 patients) and Covadonga Hospital, University of Oviedo (Oviedo, Spain; 52 patients). Patients were chosen randomly among those with detailed clinical and follow-up data to cover the entire spectrum of differentiation for tumors of follicular cell origin. All histologic diagnoses were reviewed according to established histologic criteria.⁶ Patients with UDC received palliative treatment, whereas those with differentiated thyroid carcinoma (WDC and PDC) underwent total thyroidectomy followed by

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Table 1. Oligonucleotide Primers, PCR Conditions and *ras*-Mutated Positive Control Human Cell Lines

<i>ras</i> Gene Exon	Codons; Wild-Type Sequence	Primer Sequence	Amplicon Size (bp)	PCR Cycling Conditions	MgCl ₂ Final Concentration in PCR (mM)	Positive Control Cell Line; <i>ras</i> Codon	Sequence Mutation; Amino Acid Substitution
K- <i>ras</i> exon-1	12/13; GGT/GGC	[+]5'-GACTGAATATAAACTGTGG-3' [-]5'-CTGTATCAAAGAATGGTCCT-3'	163	35 cycles: 30" at 94°C, 45" at 55°C, 45" at 73°C	2.5	ALA; 12 A549; 12 LU65; 12 SW480; 12 GLY; 13	GGT>GAT; Gly>Asp GGT>AGT; Gly>Ser GGT>TGT; Gly>Cys GGT>GTT; Gly>Val GGC>GAC; Gly>Asp
K- <i>ras</i> exon-2	61; CAA	[+]5'-GACTGTGTTTCTCCCTCT-3' [-]5'-TGGCAAATACACAAAGAAAG-3'	161	35 cycles: 30" at 94°C, 45" at 55°C, 45" at 73°C	2.5		
N- <i>ras</i> exon-1	12/13; GGT/GGT	[+]5'-GACTGAGTACAACTGGTGG-3' [-]5'-GGGCCTCACCTCTATGGTG-3'	118	35 cycles: 30" at 94°C, 45" at 55°C, 45" at 73°C	2.0	MOLT-4; 12	GGT>TGT; Gly>Cys
N- <i>ras</i> exon-2	61; CAA	[+]5'-GGTGAACCTGTTTGTGGA-3' [-]5'-ATACACAGAGGAAGCCCTCG-3'	103	35 cycles: 30" at 94°C, 45" at 55°C, 45" at 73°C	4.0	HL-60; 61	CAA>CTA; Gln>Leu
H- <i>ras</i> exon-1	12/13; GGC/GGT	[+]5'-CAGGCCCTGAGGAGCGATG-3' [-]5'-TTCGTCCACAAAATGGTCT-3'	110	35 cycles: 30" at 94°C, 45" at 60°C, 45" at 73°C	1.75	T24; 12	GGC>GTC; Gly>Val
H- <i>ras</i> exon-2	61; CAG	[+]5'-TCCTGCAGGATTCACCGG-3' [-]5'-GGTTCACCTGTACTGGTGGGA-3'	194	35 cycles: 30" at 94°C, 45" at 55°C, 45" at 73°C	2.0		

Abbreviations: PCR, polymerase chain reaction; ", seconds.

postoperative iodine treatment, according to standard clinical protocols. Forty-nine patients died as a result of disease during follow-up; all cancer survivors were observed for a median period of 84 months (range, 11 to 262 months) or until death. Processing of samples and of patient information proceeded in agreement with review board approved protocols.

DNA Extraction

DNA was isolated from 125 formalin-fixed, paraffin-embedded tumor specimens corresponding to the 107 patients with thyroid carcinoma. The presence of tumor in the samples selected for *ras* mutational analysis was verified for all patients by microscopic examination of histology sections obtained from the paraffin blocks. When necessary, tumor material was manually microdissected to increase the proportion of neoplastic cells, which always represented at least 80% of the total. Multiple specimens were analyzed from the same tumor for 14 patients, whenever areas with differing morphologic features could be dissected, or when samples for the tumor recurrence or metastases were available. DNA extraction was performed according to previously described protocols.⁷

PCR-SSCP

Tumor DNA was evaluated for point mutations at codons 12, 13, and 61 of K-, H- and N-*ras* by PCR-SSCP. PCR-SSCP was performed with procedures similar to those previously described.⁷ The primers used for amplification, PCR conditions, and the American Type Culture Collection cell lines used as positive controls are listed in Table 1. Placental DNA was included as wild-type control for each *ras* gene PCR-SSCP assay. Two microtubes without template DNA, which were covered before and after target DNA was added to the remaining tubes, were included as negative controls for each *ras* gene PCR-SSCP assay to ensure reagent purity and proper handling. For SSCP analysis, samples were analyzed with mutation detection enhancement (MDE; FMC BioProducts, Rockland, ME) gel matrices. Forty percent MDE gels were used for all amplicons with the exception of H-*ras* exon-1 amplicons, which required 50% MDE gels. After electrophoresis the gels were stained with SYBR-Green Gold nucleic acid gel stain (FMC BioProducts).

Sequence Analysis

All bands from samples exhibiting reproducible mobility shifts after independent repeat of the PCR-SSCP assay were excised, eluted, and

amplified by PCR using the same primer set and reaction conditions described above. The reamplified products were separated, purified, and analyzed by the W.M. Keck Biotechnology Resource Laboratory at Yale University using an automated Applied Biosystems 373A Stretch DNA sequencer (Perkin-Elmer, Norwalk, CT). Nucleotide sequencing from both the sense and antisense orientation was performed for confirmation. All mutated patient samples were further verified by repeating the PCR-SSCP assay.

Statistical Analysis

Only activating mutations were considered for statistical analysis. Point mutations at codons 12 or 13 of exon 1 or at codon 61 of exon 2 of H-, K- or N-*ras*; the presence of activating mutations with nucleotide transitions and transversions in any *ras* gene; or the occurrence of single and of multiple *ras*-activating mutations, as well as the type of amino acid substitution, were coded as "yes" or "no" for data analysis. When multiple specimens were genotyped from the same tumor, all *ras* mutations identified were considered for the computation. The two-tailed Fisher's exact test was used to assess the association between the altered *ras* phenotype and clinicopathologic categories. Logistic regression statistics was used to investigate the relationship between *ras* mutations, clinicopathologic parameters, tumor differentiation, and origin (Spain or United States). Logistic regression results were verified by bootstrap analysis.⁸ Disease-specific survival was analyzed using Kaplan-Meier plots and log-rank tests. Patients who died as a result of tumor were classified as uncensored, whereas those who were still alive (with or without disease) or had died as a result of unrelated causes were coded as censored. Prognostic models were devised using the Cox's proportional hazard method. Computing was performed using STATVIEW (SAS Institute Inc, Cary, NC) and GraphPad Prism (GraphPad, San Diego, CA) software.

RESULTS

Pathologic and Clinical Data

The pathologic and clinical features of the tumors are summarized in Table 2. Similar to previous descriptions,⁷ the tumors were divided into three types on the basis of their degree of differentiation. The first set consisted of well-differentiated thyroid carcinomas (16 patients from Spain; 33 patients from the

Table 2. Pathologic and Clinical Features of 107 Thyroid Tumors Analyzed for H-, K-, and N-ras Mutations

Tumor Differentiation	Sex (F/M)		Age Median, (years)	Histologic Diagnosis		Mean Size (cm)	Extrathyroid Extension		Vascular Invasion		Lymph Node Metastasis		Distant Metastases		Stage (AJCC)		Follow-Up*	
	No.	%		No.	%		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
WDC (n = 49)	37/12	75.5/24.5	49	Papillary, 30	61.2	4.1	17	34.7	28	57.1	20	40.8	7	14.3	I 17	34.7	NED 35	71.5
				Follicular, 19	38.8										II 14	28.5	AWD 6	12.2
PDC (n = 29)	16/13	55.2/44.8	56	Papillary, 14	48.3	7.8	17	58.6	27	93.1	12	41.3	17	58.6	III 16	32.7	DOC 1	2.0
				Follicular, 15	51.7										IV 2	4.1	DOD 7	14.3
															I 4	13.8	NED 11	37.9
															II 6	20.7	AWD 5	17.2
Undifferentiated (n = 29)	19/10	65.5/34.5	69	Anaplastic carcinoma		9.3	29	100	20	69.0	11	37.9	10	34.5	III 11	37.9	DOD 13	44.9
															IV 8	27.6		
															IV 29	100	DOD 29	100

Abbreviations: F, female; M, male; WDC, well-differentiated carcinoma; PDC, poorly differentiated carcinoma; NED, no evidence of disease; AWD, alive with disease; DOD, death from other causes; DOD, death from disease; AJCC, American Joint Committee on Cancer.

*Forty-nine patients died as a result of disease during follow-up; all cancer survivors were observed for a median period of 84 months (range, 11 to 262 months) or until death.

United States) of either papillary or follicular type. The second set consisted of PDCs (16 patients from Spain; 13 patients from the United States) exhibiting the features of insular carcinoma (12 patients)⁹ or consisting of neoplasms with a trabecular or solid (comedo type) growth pattern with nuclear hyperchromasia, high mitotic activity, and necrosis (poorly differentiated thyroid carcinoma not otherwise specified, 17 patients).⁶ On the basis of their morphologic features, the PDCs were also classified as papillary or follicular (Table 2). The third set included anaplastic UDCs (20 patients from Spain; nine patients from the United States). Histologic classification of thyroid carcinomas in the three groups according to their degree of differentiation successfully stratified the mortality risk for thyroid carcinoma (Fig 1A), thus justifying the validity of this approach. Differentiated thyroid carcinomas (WDC and PDC) are traditionally subclassified into papillary and follicular histotypes. There was no significant difference in survival after conventional morphologic subclassification of the differentiated carcinoma group (PDC and WDC, 78 patients) into differentiated follicular and differentiated papillary cancer (Fig 1B). Patients with oncocyctic tumors (Hürthle cell carcinomas) had a higher mortality compared with patients with other differentiated carcinomas but the difference was not statistically significant (data not shown).

Because there was a significant association between tumor origin (Spain v United States) and poorly or undifferentiated tumors ($P = .006$), tumor origin was included as a covariate in assessing the association between *ras* mutation and clinicopathologic parameters or survival (see subsection, *ras* Mutation Pattern and Correlation With Clinicopathologic Parameters).

ras Mutation Pattern and Correlation With Clinicopathologic Parameters

The SSCP pattern and sequence analysis of common types of *ras* mutations are illustrated in Figure 2. The results of H-, K- and N-*ras* mutation analysis and the correlation of *ras* mutation status with clinicopathologic features are summarized in Tables 3 and 4, respectively. *ras* mutations were identified in 35 of 107 tumors (32.7%). K-*ras* had the largest number of mutations (26 of 107 tumors, 24.3%), all of them occurring either at codon 12 or at codon 13 of exon 1. N-*ras* was mutated in nine of 107 tumors (8.4%) and H-*ras* was mutated in five of 107 tumors (4.7%). The majority of nucleotide changes were transitions (26 of 107 tumors, 24.3%). Multiple activating mutations were detected in 17 of the 35 tumors that harbored oncogenic *ras* and were restricted to PDC and UDC.

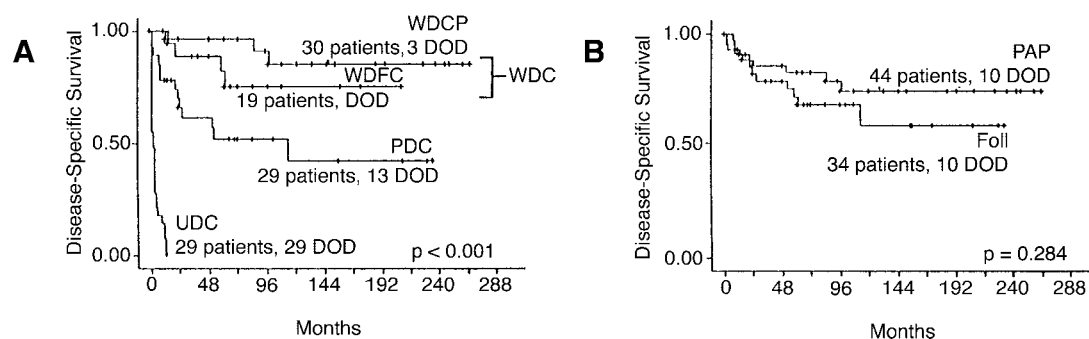


Fig 1. (A) Survival of 107 patients with thyroid carcinoma according to tumor differentiation: WDC, well-differentiated carcinoma; WDCP, well-differentiated papillary carcinoma; WDFC, well-differentiated follicular carcinoma; PDC, poorly differentiated carcinoma; UDC, undifferentiated carcinoma (log-rank test). (B) Survival of 78 patients with differentiated thyroid carcinoma according to the traditional subclassification into follicular (FOLL) and papillary (PAP) histotypes (log-rank test). DOD, dead of disease.

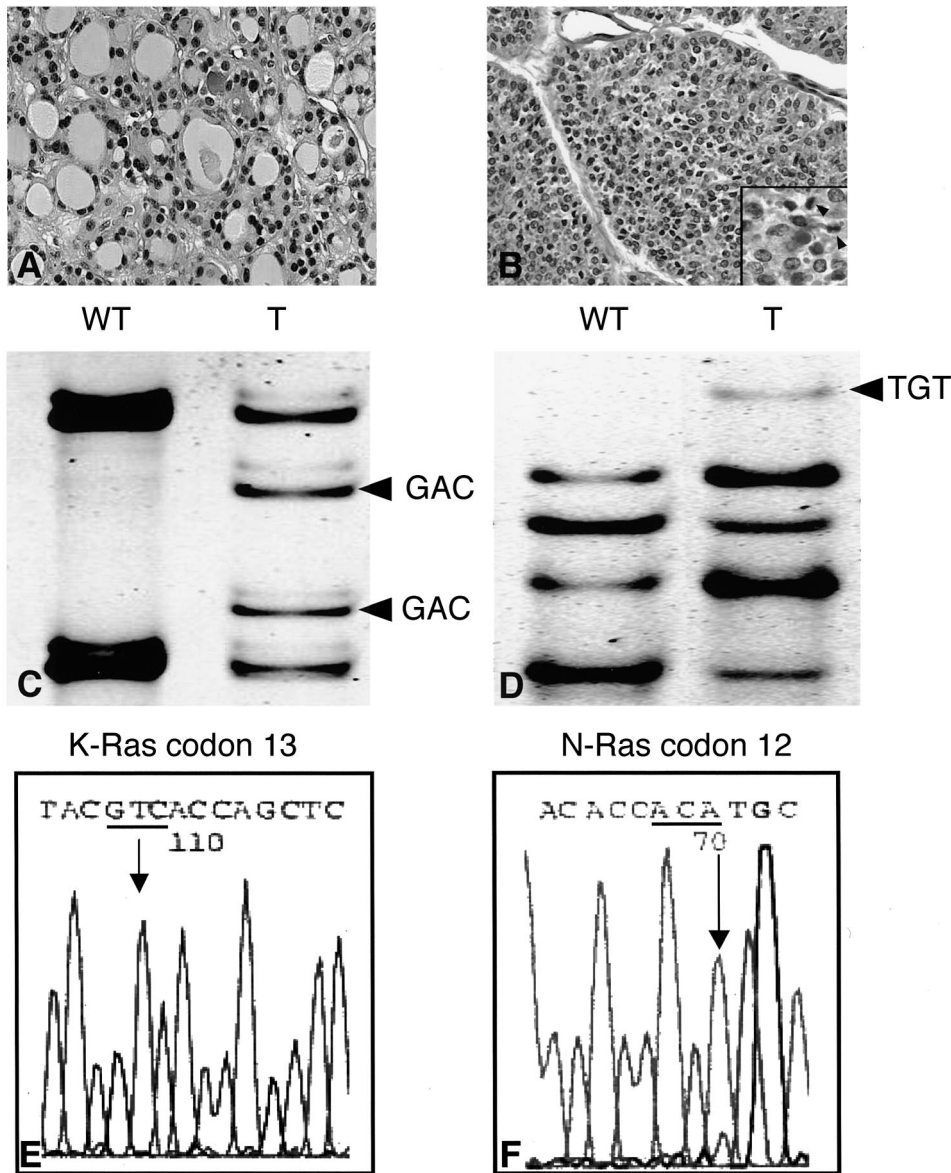


Fig 2. (A and B) Histologic appearance, (C and D) polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP) gels, (E and F) and sequencing profiles of one well-differentiated follicular carcinoma (A, C, E) and one poorly differentiated (insular) carcinoma (B, D, F). DNA sequencing demonstrates a $\text{GGC} > \text{GAC}$ (Gly>Asp) mutation (E) and a $\text{GGT} > \text{TGT}$ (Gly>Cys) mutation (F). WT, wild type; T, tumor.

Mutational analysis in the eight PDCs and six UDCs with additional DNA samples from tumor recurrence, metastases, and areas with different histologic appearance demonstrated that *ras* mutations segregated with the less-differentiated portions of the tumor and that the type and pattern of *ras* mutations was consistent with that identified in the primary lesion.

Amino acid changes at the *ras* GTP-binding domain (corresponding to codons 12 and 13 of exon 1) involved replacement of glycine with aspartic acid (30 mutations), cysteine (13 mutations), serine (eight mutations), alanine (four mutations), and valine (three mutations; Table 3). Amino acid changes at the *ras* GTPase domain (corresponding to codon 61 of exon 2) involved replacement of glutamine with leucine (six mutations), histidine (one mutation), and proline (one mutation; Table 3). All activating mutations at codon 13 of *K-ras* (17 mutations) and of *H-ras* (two mutations) were G-A transitions resulting in replacement of glycine with aspartic acid. All activating mutations at

codon 12 of *N-ras* (six mutations) were G-T transversions resulting in replacement of glycine with cysteine. Nucleotide changes not resulting in replacement of amino acid residues (silent *ras* mutations) were only identified at the *ras* GTP-binding domain (codons 12 or 13 of exon 1) and, with the exception of four patients, always coexisted with activating mutations (Table 3).

ras mutations were present in four of 49 (8.2%) WDCs (two of 30 [6.7%] of well-differentiated papillary carcinomas and two of 19 [10.5%] of well-differentiated follicular carcinomas), in 16 of 29 (55.2%) PDCs, and in 15 of 29 (51.7%) UDCs, with a significant association between *ras* mutation and poorly or undifferentiated tumor phenotypes ($P < .001$, χ^2 test for trend). Among the 78 patients of the differentiated carcinoma group (PDC and WDC), *ras* mutations were identified in 11 of 44 (25%) thyroid carcinomas of papillary histotype and in nine of 34 (26%) of follicular histotype. There was no significant

Table 3. Nucleotide Changes and Amino Acid Substitutions in the Thyroid Carcinomas With Mutated *ras*

Well-Differentiated Carcinomas, Papillary		Well-Differentiated Carcinomas, Follicular		Poorly Differentiated Carcinomas, Papillary	
ID	Mutations	ID	Mutations	ID	Mutations
25	H- <i>ras</i> codon 13: GGT>GAT (Gly>Asp)	37	K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp)	55	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp) N- <i>ras</i> codon 61: CAA>CTA (Gln>Leu)
132	N- <i>ras</i> codon 61: CAA>CTA (Gln>Leu)	59	K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp)	56	K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp) N- <i>ras</i> codon 12: GGT>TGT (Gly>Cys) N- <i>ras</i> codon 61: CAA>CTA (Gln>Leu)
				65	N- <i>ras</i> codon 61: CAA>CTA (Gln>Leu)
				69	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp) K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp)
				70	N- <i>ras</i> codon 13: GGT>TGT (Gly>Cys)
				71	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp); GGT>GCT (Gly>Ala); GGT>TGT (Gly>Cys)
					K- <i>ras</i> codon 13: GGC>GGT (silent mutation)
				72	K- <i>ras</i> codon 12: GGT>AGT (Gly>Ser) K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp)
				78	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp) K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp)
				79	K- <i>ras</i> codon 12: GGT>AGT (Gly>Ser) K- <i>ras</i> codon 13: GGC>GGT (silent mutation)

correlation between the type of *ras* mutations and whether a differentiated thyroid tumor was classified morphologically as papillary or follicular carcinoma, or whether it exhibited oncogenic features. Samples originating from Spain had an overall higher prevalence of activating *ras* mutation because of the higher proportion of PDC and UDC among the Spanish patients. *ras* mutations were not associated with patient sex, age, or the presence of lymph node metastases.

In general, *ras* mutations were associated not only with histologic features (ie, loss of tumor differentiation) but also with clinicopathologic parameters indicative of aggressive behavior, such as large tumor size and vascular invasion (Table 4), reflecting the strong link between *ras* mutations and poorly or undifferentiated thyroid tumors. To test whether *ras* mutations may have a direct influence on the biologic behavior of the tumors, for each of the four clinicopathologic categories significantly associated with the *ras* mutation status in Table 4 (ie, tumor size, extrathyroidal extension, vascular invasion, and distant metastases) logistic regression models were analyzed with each individual category mentioned above as a dependent variable. Specific *ras* mutation patterns were entered as independent covariates in separate models, together with tumor differentiation and origin (Spain *v* United States). This type of analysis demonstrated that K-*ras* mutations, specifically those involving codon 13, are independently associated with distant metastases, either at presentation or diagnosed during the follow-up period (relative risk, 4.16; $P = .018$).

ras Mutations and Disease-Specific Survival

Twenty-six of 35 patients (74.3%) with *ras*-mutated tumors died as a result of disease as opposed to 23 of 72 patients (31.9%) with tumors lacking the mutations (Fig 3). In particular, poor survival was associated with the presence of activating mutations in any of the *ras* genes (relative risk, 3.37; $P < .001$),

K-*ras* mutations (relative risk, 2.70; $P < .001$), K-*ras* codon 13 (relative risk, 2.15; $P = .022$), or N-*ras* (relative risk, 2.43; $P = .030$). Both patients with the *ras*-mutated tumors illustrated in Figure 2 died as a result of disease during follow-up. H-*ras* mutations did not significantly correlate with poor survival in this series. Because of the high prevalence of *ras* mutations in PDC and UDC, the association with poor survival was not independent of tumor differentiation and stage. There was no significant difference in survival between the patients diagnosed and treated in Spain and those from the United States after adjustment for tumor differentiation and stage ($P = .380$).

Given the uniformly fatal outcome of patients with UDC (all of them were dead as a result of disease within 1 year of diagnosis; Table 2) survival analysis was also performed, after exclusion of UDC patients, on the remaining differentiated thyroid carcinomas (WDC and PDC). The results, which are illustrated in Figure 4, demonstrate that the *ras* mutation status is associated with tumor-related death. Table 5 summarizes the results of multivariate analyses. Specific types of *ras* mutations were entered as covariates in separate models, together with tumor differentiation (WDCs *v* PDCs), tumor stage, and origin (Spain *v* United States). As expected, tumor stage was always an independent predictor of survival, with a relative risk ranging from 12 to 18 for stage 3 tumors and from 12 to 44 for stage 4 tumors. *ras* mutations in general, as well as the specific *ras* mutation patterns shown in Table 5, were independently associated with survival and were more powerful predictors of outcome than the morphologic diagnosis of poor tumor differentiation. Patients with K-*ras* codon 13 mutated tumors, with tumors harboring Gly>Asp substitutions (Fig 4), and with tumor mutations caused by nucleotide transitions (not shown) had similar survival curves because all of the patients with K-*ras* codon 13 mutations featured second nucleotide G-A transitions in codon 13, resulting in Gly>Asp substitutions. Conversely, K-*ras* codon 13 was mutated in the majority of the differentiated carcino-

Table 3. Nucleotide Changes and Amino Acid Substitutions in the Thyroid Carcinomas With Mutated *ras* (Continued)

Poorly Differentiated Carcinomas, Follicular		Undifferentiated Carcinomas	
ID	Mutations	ID	Mutations
8	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp) K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp) H- <i>ras</i> codon 61: CAG>CTG (Gln>Leu); CAG>CAC (Gln>His)	15	H- <i>ras</i> codon 12: GGC>GGA (silent mutation) H- <i>ras</i> codon 13: GGT>GAT (Gly>Asp); GGT>GGC (silent mutation)
68	K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp)	17	K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp)
73	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp); GGT>GCT (Gly>Ala) K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp); GGC>GGT (silent mutation)	83	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp); GGT>TGT (Gly>Cys) K- <i>ras</i> codon 13: GGC>GGT (silent mutation)
74	K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp)	85	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp); GGT>AGT (Gly>Ser) K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp); GGC>GGT (silent mutation)
76	K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp)	87	K- <i>ras</i> codon 12: GGT>GTT (Gly>Val); GGT>TGT (Gly>Cys) K- <i>ras</i> codon 13: GGC>GGT (silent mutation)
81	K- <i>ras</i> codon 12: GGT>AGT (Gly>Ser)	88	K- <i>ras</i> codon 12: GGT>GCT (Gly>Ala); GGT>GTT (Gly>Val)
82	K- <i>ras</i> codon 13: GGC>GGT (silent mutation) H- <i>ras</i> codon 61: GAG>GGG (Gln>Pro)	89	K- <i>ras</i> codon 12: GGT>TGT (Gly>Cys) K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp); GGC>GGT (silent mutation) N- <i>ras</i> codon 12: GGT>TGT (Gly>Cys)
5	H- <i>ras</i> codon 12: GGC>GGA (silent mutation)	90	N- <i>ras</i> codon 12: GGT>TGT (Gly>Cys)
77	H- <i>ras</i> codon 13: GGT>GGC (silent mutation) H- <i>ras</i> codon 12: GGC>GGA (silent mutation) H- <i>ras</i> codon 13: GGT>GGC (silent mutation)	91	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp); GGT>TGT (Gly>Cys); GGT>AGT (Gly>Ser) K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp); GGC>GGT (silent mutation) N- <i>ras</i> codon 12: GGT>TGT (Gly>Cys) H- <i>ras</i> codon 12: GGC>GGA (silent mutation) H- <i>ras</i> codon 13: GGT>GGC (silent mutation)
		92	H0 <i>ras</i> codon 12: GGC>AGC (Gly>Ser)
		93	K- <i>ras</i> codon 12: GGT>TGT (Gly>Cys); GGT>AGT (Gly>Ser) K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp); GGC>GGT (silent mutation)
		95	K- <i>ras</i> codon 12: GGT>GTT (Gly>Val)
		96	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp)
		97	N- <i>ras</i> codon 12: GGT>TGT (Gly>Cys) N- <i>ras</i> codon 13: GGT>GCT (Gly>Ala) N- <i>ras</i> codon 61: CAA>CTA (Gln>Leu)
		100	K- <i>ras</i> codon 12: GGT>GAT (Gly>Asp); GGT>TGT (Gly>Cys); GGT>AGT (Gly>Ser) K- <i>ras</i> codon 13: GGC>GAC (Gly>Asp); GGC>GGT (silent mutation)
		12	H- <i>ras</i> codon 12: GGC>GGA (silent mutation) H- <i>ras</i> codon 13: GGT>GGC (silent mutation)
		102	H- <i>ras</i> codon 12: GGC>GGA (silent mutation) H- <i>ras</i> codon 13: GGT>GGC (silent mutation)

mas with Gly>Asp substitutions (11 of 14 patients) and with nucleotide transition mutations (11 of 16 patients). Tumor origin did not influence survival. Similarly, histologic classification of differentiated thyroid tumors as papillary or follicular did not have a statistical influence on the association between *ras* mutation status and survival.

DISCUSSION

Activating H-, K-, and N-*ras* mutations represent the most common type of abnormality of a dominant oncogene in human

cancer and have been identified in many different types of tumors, with specificity and type of mutation varying in relation to the tumor type.^{1,10} Numerous studies have addressed the relationship between *ras* mutations and the clinicopathologic features of the tumors harboring the mutation. Several studies, including a prospective study¹¹ and large meta-analyses,^{12,13} have shown that *ras* mutations are associated with poor prognosis in colorectal adenocarcinoma and that different gene mutations have different prognostic impact.¹¹⁻¹³ Both prospective¹⁴ and retrospective^{15,16} analyses have shown that K-*ras* mutations

Table 4. *ras* Mutations and Clinicopathologic Parameters in 107 Thyroid Carcinomas

<i>ras</i> Mutation Type	Tumor Type								Tumor Size ≥ 6 cm		Extra Thyroidal Tumor Extension*			Vascular Invasion			Distant Metastases			
	Total No. of Tumors	%	WDC		PDC		UDC		No.	%	P	No.	%	P	No.	%	P	No.	%	P
H-, K-, or N- <i>ras</i> , n = 35	32.7	4	8.2	16	55.2	15	51.7	<.001	30	50.8	<.001	27	44.3	.004	30	40.0	.014	17	50.0	.014
K- <i>ras</i> , n = 26	24.3	2	4.1	13	44.8	11	37.9	<.001	24	40.7	<.001	19	31.1	.070	24	32.0	.006	15	44.1	.002
K- <i>ras</i> codon 13, n = 17	15.9	2	4.1	9	31.0	6	20.7	.023	15	25.4	.003	4	6.5	NS	17	22.6	.003	11	32.3	.003
N- <i>ras</i> , n = 9	8.4	1	2.0	4	13.8	4	13.8	.050	7	11.8	NS	1	1.6	NS	8	10.6	NS	4	11.7	NS
H- <i>ras</i> , n = 5	4.7	1	2.0	2	6.9	2	6.9	NS	2	3.4	NS	4	6.5	NS	3	4.0	NS	2	5.8	NS
Nucleotide transitions† n = 26	24.3	3	6.12	13	44.8	10	34.5	<.001	22	37.3	<.001	18	29.5	.176	2	2.6	.085	14	41.2	.008
Single activating mutations† n = 18	16.8	4	8.2	8	27.6	6	20.7	.096	15	25.4	.009	14	22.9	.068	15	20.0	NS	10	29.4	.026
Gly>Asp in exon 1‡ n = 23	21.5	3	6.1	11	37.9	9	31.0	.003	19	32.2	.004	16	26.2	NS	20	26.6	.070	12	35.3	.024
Total n = 107	100	49	100	29	100	29	100		59	100		61	100		75	100		34	100	

NOTE. Only data on activating *ras* mutations are included; the correlation between *ras* mutations and degree of tumor differentiation was analyzed with the χ^2 test for trend, the remaining data with the Fisher's exact test. P values between .05 and .2 are in italics; they are not reported if $\geq .2$.

Abbreviations: WDC, well-differentiated carcinoma; PDC, poorly differentiated carcinoma; UDC, undifferentiated tumors; NS, not significant.

*Confirmed histologically for all patients.

†In any H-, K- or N-*ras*.

‡Mutations involving Gly>Asp in the GTP binding domain (corresponding to exon 1) of H-, K-, or N-*ras*.

are associated with poor prognosis in non-small-cell lung carcinoma. The type of K-*ras* mutation may also influence survival in pancreatic adenocarcinoma,¹⁷ whereas N-*ras* mutations are associated with failure to achieve complete remission in acute myeloid leukemia.¹⁸

Constitutive activation of all three *ras* oncogenes (H-, K-, and N-*ras*) is known to occur among tumors that originated from the follicular epithelium of the thyroid gland.¹⁹ However, there are significant discrepancies related to the overall frequency of *ras* mutations (ranging from 7% to 62%)^{20,21} and their prevalence in

specific thyroid tumors. No consistent relationship between tumor histotype or biologic behavior and one particular pattern of *ras* activation can be inferred from a review of the literature. Although it is difficult to explain this lack of consistency, the mutation screening methods, the selection of patients, and the design of individual studies are critical to identify specific associations between *ras* mutational status and clinical or pathologic parameters. We have used PCR-SSCP, a technique that, despite its high sensitivity²² and its widespread use for mutation detection in human cancer, has only infrequently been applied to

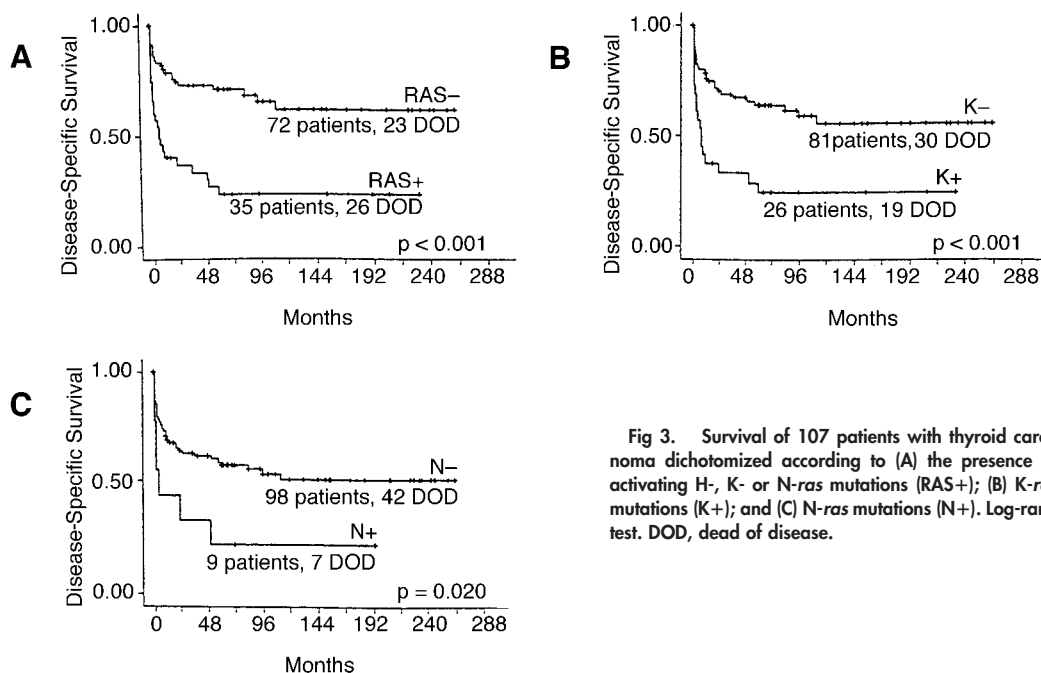


Fig 3. Survival of 107 patients with thyroid carcinoma dichotomized according to (A) the presence of activating H-, K- or N-*ras* mutations (RAS+); (B) K-*ras* mutations (K+); and (C) N-*ras* mutations (N+). Log-rank test. DOD, dead of disease.

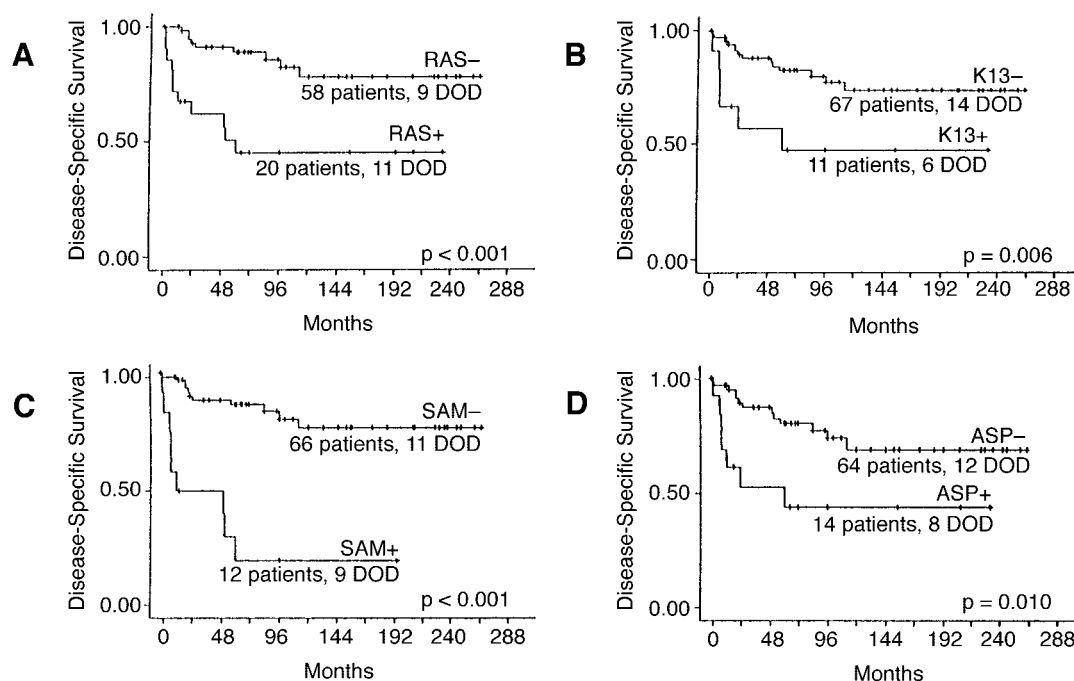


Fig 4. Survival of 78 patients with differentiated thyroid carcinoma (WDC, well-differentiated carcinoma; PDC, poorly differentiated carcinoma) dichotomized according to the presence of (A) activating H-, K- or N-ras mutations (RAS+); (B) K-ras codon 13 mutations (K13+); (C) single activating mutations in any ras gene (SAM+); and (D) mutations involving Gly>Asp amino acid substitutions (ASP+). Log-rank test. DOD, dead of disease.

the study of thyroid neoplasms.^{21,23} We have also selected tumors that include the full spectrum of differentiation observed in thyroid cancer of follicular cell derivation with a large number of patients—the largest series so far analyzed for *ras* mutations—to allow for meaningful statistical analysis.

This study demonstrates that *ras* mutations define a subset of thyroid carcinoma characterized by aggressive behavior. This is indicated by the close relationship between oncogenic *ras* and the loss of those histologic features that characterize well-

differentiated thyroid tumor phenotypes.⁶ Remarkably, oncogenic *K-ras* not only correlates with the loss of tumor differentiation but also with the presence of distant metastases, independent of tumor differentiation. This is consistent with the *ras* role in modulating cell motility and invasiveness, and with the recent observation that *K-ras* mutations are detectable in the large majority of early metastatic deposits in the bone marrow of patients with colonic adenocarcinoma.²⁴ Potentially relevant for patient management is the finding that *ras* mutations are asso-

Table 5. *ras* Mutation and Survival in 78 Differentiated Thyroid Carcinoma (WDC and PDC)

<i>ras</i> Activation Type	Univariate*			Multivariate†			
	RR	95% Confidence Interval	P	Prognostic Model	RR	95% Confidence Interval	P
H-, K-, or N- <i>ras</i>	5.00	2.07 to 12.10	< .001	H-, K-, or N- <i>ras</i>	4.69	1.32 to 16.69	.016
K- <i>ras</i> codon 13	3.51	1.34 to 9.15	.010	PDC	1.97	0.53 to 7.34	NS
				K- <i>ras</i> codon 13	4.03	1.17 to 13.87	.027
Nucleotide transitions‡	4.32	1.78 to 10.40	.001	PDC	2.59	0.75 to 8.76	.132
				Nucleotide transitions‡	4.01	1.24 to 12.99	.020
Single activating mutations‡	8.03	3.28 to 19.60	< .001	PDC	2.45	0.72 to 8.28	.149
				Single activating mutations‡	5.60	1.58 to 19.78	.007
Gly>Asp in exon 1§	3.13	1.25 to 7.87	.015	PDC	3.21	0.99 to 10.42	.053
				Gly>Asp in exon 1§	4.61	1.37 to 15.38	.013
				PDC	2.58	0.79 to 8.45	.118

NOTE. Only data on activating *ras* mutations are included. P values between .05 and .2 are in italics; they are not reported if ≥ .2.

Abbreviations: WDC, well-differentiated carcinoma; PDC, poorly differentiated carcinoma; RR, relative risk of tumor-related death; NS, not significant.

*The relative risk, 95% confidence interval and P value of tumor related death according to tumor differentiation (well versus poorly differentiated carcinomas) were 4.60, 1.82 to 11.58, and .001; the relative risk, 95% confidence interval and P value of tumor related death according to stage (stage 1 and 2 v stage 3 and 4) were 8.63, 2.52 to 29.51, < .001, respectively. Ten of 44 patients with differentiated thyroid tumor of papillary morphology died of disease (22.7%) as opposed to 10 of 34 patients with tumors of follicular histotype (29.4%), but there was no significant correlation with poor outcome after univariate analysis (P = .289).

†Multivariate analysis was performed using the type of *ras* mutation, tumor differentiation (well v poorly differentiated carcinomas), tumor stage, and origin (Spain v United States) as independent covariates. As expected, tumor stage was always an independent predictor of survival with a relative risk ranging from 12 to 18 for stage 3 tumors and from 12 to 44 for stage 4 tumors. The origin of the tumor was not an independent predictor of survival.

‡In any H-, K- or N-*ras*.

§Mutations involving Gly>Asp in the guanosine triphosphate-binding domain (corresponding to exon 1) of H-, K- or N-*ras*.

ciated with poor prognosis among differentiated carcinomas (the WDC and PDC tumor group) independent of tumor stage and of whether the tumor is subclassified morphologically as well or poorly differentiated, papillary or follicular. As expected, stage exhibited the strongest correlation with survival but the *ras* mutation status was a more powerful indicator of outcome than the histologic diagnosis of PDC. Patients with UDC, because of the rapidly fatal outcome of this tumor type, represented a bias for the survival analysis that justified their separation from the rest of the tumors. Indeed, UDC has long been recognized as a distinctive type of thyroid malignancy characterized by complete loss of tumor differentiation and an aggressive behavior resulting in the patient death as a result of uncontrollable tumor growth in the neck.⁶

The vast majority of the remaining types of thyroid carcinoma are indolent tumors, but a small minority of them can be difficult to control and may ultimately result in patient death.²⁵ These neoplasms include a rather heterogeneous morphologic spectrum and often exhibit the histologic features of PDC.⁶ Unlike the morphologic criteria that define UDC, those used by different pathologists for the diagnosis of poorly differentiated carcinomas are not always comparable. The results of this study indicate that *ras* mutation analysis may provide an objective tool to identify those thyroid tumors which, apart from the fatal but rare UDC, are also associated with patient death.

Consistent with previous studies,^{19,26} we have identified activating mutations of all three *ras* genes in thyroid cancer. We have demonstrated for the first time that H-, K- or N-*ras* may occur in each of the three differentiation types of thyroid carcinoma. Similar to what has been shown for other tumor types with a high prevalence of oncogenic *ras*, such as colonic¹¹⁻¹³ and pancreatic¹⁷ adenocarcinoma, specific mutation patterns may have a different influence on metastatic potential and survival. K-*ras* codon 13 mutations (all of which involved second nucleotide G-A transitions in codon 13 resulting in Gly>Asp substi-

tion) were a marker for distant metastases and, among patients with differentiated thyroid cancer, poor survival. This molecular change was present in the majority of patients with nucleotide transitions and Gly>Asp mutations, which explains the influence that both alterations have on survival. K-*ras* codon 13 Gly>Asp mutations have been associated with an increased risk of disease recurrence in a prospective study of colonic adenocarcinoma.¹¹ The K-*ras* codon 12 Gly>Val mutation, which has been associated with poor survival in colon cancer,¹³ was present in three of our patients, all of whom had UDC, indicating that it may represent a marker for tumor aggressiveness in thyroid cancer as well. Single activating *ras* mutations were a marker for poor survival in the differentiated carcinoma group and were present in the majority of the patients with PDC who died as a result of disease (data not shown), whereas multiple mutations did not have an independent influence on survival.

The results of this study demonstrate a clear link between *ras* mutations and poor prognosis. They therefore provide a rational basis for treating thyroid cancer with chemotherapeutic agents that target *ras*, such as farnesyl transferase inhibitors. The greatest potential for detecting a benefit in clinical trials with these drugs should be in patients with tumors that are highly likely to harbor mutated *ras*,²⁷ such as poorly and undifferentiated thyroid cancers. The recent demonstration that the farnesyl transferase inhibitor manumycin A inhibits UDC growth both in vitro and in nude mouse xenografts²⁸ is consistent with this hypothesis.

In summary, we show that *ras* mutations are a marker for aggressive thyroid cancer behavior and poor outcome. Although additional investigations and particularly prospective studies are necessary to elucidate further the relationship between *ras* oncogene activation and thyroid neoplasia, our results indicate that *ras* genotyping may be of significant value as a prognostic indicator and may provide the rationale for novel treatment modalities.

REFERENCES

- Bos JL: Ras oncogenes in human cancer: A review. *Cancer Res* 49:4682-4689, 1989
- Wright PA, Williams ED, Lemoine NR, et al: Radiation-associated and 'spontaneous' human thyroid carcinomas show a different pattern of ras oncogene mutation. *Oncogene* 6:471-473, 1991
- Challeton C, Bounacer A, Du Villard JA, et al: Pattern of ras and gsp oncogene mutations in radiation-associated human thyroid tumors. *Oncogene* 11:601-603, 1995
- Suchy B, Waldmann V, Klugbauer S, et al: Absence of RAS and p53 mutations in thyroid carcinomas of children after Chernobyl in contrast to adult thyroid tumours. *Br J Cancer* 77:952-955, 1998
- Hara H, Fulton N, Yashiro T, et al: N-ras mutation: An independent prognostic factor for aggressiveness of papillary thyroid carcinoma. *Surgery* 116:1010-1016, 1994
- Rosai J, Carcangiu ML, DeLellis RA: Tumors of the thyroid gland, in Atlas of Tumor Pathology (series 3, fascicle 5). Washington, DC, Armed Forces Institute of Pathology, 1992
- Garcia-Rostan G, Camp RL, Herrero A, et al: b-catenin dysregulation in thyroid neoplasms: Down-regulation, aberrant nuclear expression and *ctnmb1* exon 3 mutations are markers for aggressive tumor phenotypes and poor prognosis. *Am J Pathol* 158:987-996, 2001
- Efron B, Tibshirani RJ, An Introduction to the Bootstrap. London, United Kingdom, Chapman & Hall, 1993
- Carcangiu ML, Zampi G, Rosai J: Poorly differentiated ("insular") thyroid carcinoma: A reinterpretation of Langhans' "wuchernde struma." *Am J Surg Pathol* 8:655-668, 1984
- Capella G, Cronauer-Mitra S, Peinado MA, et al: Frequency and spectrum of mutations at codons 12 and 13 of the C-K-ras gene in human tumors. *Environ Health Perspect* 93:125-131, 1991
- Cerottini JP, Caplin S, Saraga E, et al: The type of K-ras mutation determines prognosis in colorectal cancer. *Am J Surg* 175:198-202, 1998
- Andreyev HJ, Norman AR, Cunningham D, et al: Kirsten ras mutations in patients with colorectal cancer: The multicenter "RASCAL" study. *J Natl Cancer Inst* 90:675-684, 1998
- Andreyev HJ, Norman AR, Cunningham D, et al: Kirsten ras mutations in patients with colorectal cancer: The 'RASCAL II' study. *Br J Cancer* 85:692-696, 2001
- Nelson HH, Christiani DC, Mark EJ, et al: Implications and prognostic value of K-ras mutation for early-stage lung cancer in women. *J Natl Cancer Inst* 91:2032-2038, 1999
- Kwiatkowski DJ, Harpole DH Jr, Godleski J, et al: Molecular pathologic substaging in 244 stage I non-small-cell lung cancer patients: Clinical implications. *J Clin Oncol* 16:2468-2477, 1998

16. Broermann P, Junker K, Brandt BH, et al: Trimodality treatment in stage III nonsmall cell lung carcinoma: Prognostic impact of K-ras mutations after neoadjuvant therapy. *Cancer* 94:2055-2062, 2002
17. Kawesha A, Ghaneh P, Andren-Sandberg A, et al: K-ras oncogene subtype mutations are associated with survival but not expression of p53, p16(INK4A), p21(WAF1), cyclin D1, erbB-2 and erbB-3 in resected pancreatic ductal adenocarcinoma. *Int J Cancer* 89:469-474, 2000
18. Kiyoi H, Naoe T, Nakano Y, et al: Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood* 93:3074-3080, 1999
19. Lemoine NR, Mayall ES, Wyllie FS, et al: Activated ras oncogenes in human thyroid cancers. *Cancer Res* 48:4459-4463, 1988
20. Horie H, Yokogoshi Y, Tsuyuguchi M, et al: Point mutations of ras and Gs alpha subunit genes in thyroid tumors. *Jpn J Cancer Res* 86:737-742, 1995
21. Pilotti S, Collini P, Mariani L, et al: Insular carcinoma: A distinct de novo entity among follicular carcinomas of the thyroid gland. *Am J Surg Pathol* 21:1466-1473, 1997
22. Emanuel JR, Damico C, Ahn S, et al: Highly sensitive nonradioactive single strand conformational polymorphism: Detection of Ki-ras mutations. *Diagn Mol Pathol* 5:260-264, 1996
23. Ezzat S, Zheng L, Kolenda J, et al: Prevalence of activating ras mutations in morphologically characterized thyroid nodules. *Thyroid* 6:409-416, 1996
24. Solakoglu O, Maierhofer C, Lahr G, et al: Heterogeneous proliferative potential of occult metastatic cells in bone marrow of patients with solid epithelial tumors. *Proc Natl Acad Sci U S A* 99:2246-2251, 2002
25. Harness JK, McLeod MK, Thompson NW, et al: Deaths due to differentiated thyroid cancer: A 46-year perspective. *World J Surg* 12:623-629, 1988
26. Manenti G, Pilotti S, Re FC, et al: Selective activation of ras oncogenes in follicular and undifferentiated carcinomas. *Eur J Cancer* 30:987-993, 1994
27. Rowinsky EK, Windle JJ, Von Hoff DD: Ras protein farnesyltransferase: A strategic target for anticancer therapeutic development. *J Clin Oncol* 17:3631-3652, 1999
28. Yeung S-C J, Xu G, Pan J, et al: Manumycin enhances the effect of paclitaxel on anaplastic thyroid carcinoma cells. *Cancer Res* 60:650-655, 2000