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Selective Activation of *ras* Oncogenes in Follicular and Undifferentiated Thyroid Carcinomas

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A total of 96 tumour samples (88 primary tumours and 8 nodal metastases) from 88 patients with thyroid adenomas and carcinomas were investigated for *ras* gene mutations using polymerase chain reaction, oligonucleotide probing and sequencing. Neither the 19 adenomas nor the 31 papillary carcinomas analysed harboured point mutations. In our cases, mutations in all three *ras* oncogenes were found in follicular carcinomas (five out of 21) and in the less differentiated thyroid tumour: poorly differentiated carcinomas (three out of 11) and undifferentiated carcinomas (one out of five). Finally, mutated *ras* oncogenes had a significant association with the appearance of haematogenous (particularly bone) metastases, suggesting a role of *ras* genes activation in the metastatic capability of these tumours.

Key words: polymerase chain reaction, DNA mutational analysis, oligonucleotide probes, genes, *ras*, thyroid neoplasms, neoplasm metastases, prognosis
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INTRODUCTION

POINT MUTATIONS leading to the activation of the transforming activity of *ras* genes (H-*ras*1, K-*ras*2, N-*ras*) have been identified in a wide spectrum of solid and haematological malignancies (for review see Bos [1]). The activating mutations occur in codons 12–13 and 61, i.e. two regions which play an important role in the *ras* p21 GTP-binding and GTPase activity, respectively (for review see [2]). Several reports indicate significant differences in the mutation frequency of *ras* genes in different tumour types. For example, mutations of *ras* genes are frequently detected in

colon and pancreas carcinomas [3, 4], whereas they are rare in mammary tumours [5, 6]. The activation of a particular member of the *ras* gene family (H-, K- or N-) also shows a sort of selective preference for a given tumour type. Notably, H-*ras*1 mutated in urinary tract tumours [7], K-*ras*2 in lung, colon and exocrine pancreas carcinomas [3, 4, 8] and N-*ras* in myeloid and lymphoid malignancies [9, 10].

In the case of tumours derived from the thyroid follicular epithelium, activation of all three *ras* oncogenes has been reported [11, 12]. However, the published data of the overall frequency of *ras* oncogenes mutation in thyroid tumours, and of their occurrence in each histotype, show some discrepancies between the different laboratories [11–15]. In fact, the rate of *ras* mutation in papillary carcinomas (PC) varies from 0 to 60% [12, 14]. A variable frequency of *ras* mutation has also been reported in both follicular adenomas (FA) and follicular carcinomas (FC) (FA from 17 to 85%; FC from 10 to 52%) and, in addition, it has been associated with different amounts of iodine intake [12, 14, 15].

PC and FC of the thyroid gland originate from the follicular epithelium, and belong to the well differentiated group of thyroid carcinomas. In spite of their common origin, the PC and FC are different entities in terms of biological aggressiveness and clinical behaviour [16]. Therefore, it seems reasonable to assume that different genetic alterations could be associated with each of the two different histotypes. In keeping with this concept, we have reported a frequent activation (50% of the cases) of two tyrosine kinase oncogenes, RET/PTC and TRK, in PC, whereas their activation was never observed in FC [17, 18].

In this study, a panel of 64 fresh and 37 paraffin-embedded specimens (including five samples also analysed in the corresponding snap-frozen tissue), representing benign and malignant epithelial thyroid tumours, including a number of samples that had been previously analysed for the activation of other oncogenes, were investigated for the presence of *ras* gene mutation. With this analysis we wanted to verify: (i) the frequency of *ras* gene mutations in the different types of thyroid epithelial tumours; (ii) the correlation between *ras* gene mutations and the activation of oncogenes with an associated tyrosine kinase activity (RET/PTC and TRK); (iii) the correlation between the presence of *ras* mutation and the clinical outcome.

MATERIALS AND METHODS

Tumours

All the thyroid tumours were collected in the Istituto Nazionale Tumori (Milan, Italy). Sixty-four fresh tissue samples of primary thyroid tumours were obtained from 56 patients; in 8 cases nodal metastases were also available. The specimens were frozen in liquid nitrogen and stored at −80°C until they were used. 37 additional cases were from Bouin-fixed, paraffin-embedded blocks. For five tumours, there were both fresh surgical and paraffin-embedded specimens. The histological diagnoses of the thyroid tumours, according to WHO [19] and subsequent updating [20–26] were as follows: 19 FA, 21 FCs, 11 poorly differentiated carcinomas (PDC), 5 undifferentiated carcinomas (UC) and 31 PCs.

The 19 FA comprised seven microfollicular or trabecular solid variants, 10 macrofollicular or normofollicular variants, and two of the oxyphilic variant [19].

FC were represented by five minimally invasive and 16 widely invasive carcinomas according to WHO [19].

The 11 PDC were made up of five insular carcinomas [21], two poorly differentiated carcinomas according to Sakamoto and colleagues [20], two oxyphilic carcinomas [23, 25], one tall cell papillary carcinoma [22] and one trabecular carcinoma [24].

UC consisted of 2 cases of the fibrosarcoma-like, 2 of giant cell variant and 1 squamous cell carcinoma [19].

Microscopically PC consisted of the following variants according to WHO, [19] and subsequent updatings [26]: eight microcarcinomas, nine follicular, one diffuse follicular [26] and 13 not otherwise specified.

DNA preparation

High molecular weight DNA was prepared from frozen tumours following standard procedures. In the cases of Bouin-fixed, paraffin-embedded tissues, tumour tissue was taken by punching the paraffin block in three selected areas with a 20 gauge needle. Then, samples were deparaffinised by adding 500 μl of xylene, washed with 95% ethanol and desiccated. Pellets were destained by several changes of 1 M Tris–HCl pH 8.3, finely fragmented and resuspended in 100 μl of 1 × PCR buffer (1 × PCR buffer = 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin). For each polymerase chain reaction (PCR), 10 μl of this suspension were used.

Preparation of oligomer

Oligonucleotides (19–20 mer) were synthesised on an Applied Biosystem model 380A. We used oligonucleotides encoding PCR primers, and wild-type or mutated *ras* sequences around codons 12–13 and 61, described by Verlaan-de-Vries and colleagues [27]. Oligomers used as primer for the direct PCR sequencing were purified on 20% acrylamide gels, those used as probes were end-labelled using [^{32}P]ATP and T4-polynucleotide kinase (New England Biolabs, Beverly, Massachusetts, U.S.A.).

PCR amplification

About 0.5 μg of genomic DNA from thyroid tumours or 10 μl of the resuspended paraffin-embedded tumour samples were mixed in 50 μl of PCR buffer, containing an appropriate $MgCl_2$ concentration in order to obtain a low level of non-specific PCR products (1 mM for H-*ras*1 61 and K-*ras*2 61; 1.5 mM for H-*ras*1 12, N-*ras* 12 and N-*ras* 61; 2 mM for K-*ras*2 12). After heating the samples at 100°C for 2 min the PCR was started by adding 50 μl of PCR mix [final concentration: 1 × PCR buffer with the appropriate $MgCl_2$ concentration, 0.2 mM of each dNTPs, 30 pmoles of each primers and 2.5 U of *Taq* polymerase (Perkin-Elmer/Cetus, Norwalk, Connecticut, U.S.A.)]. The DNA samples were subjected to 25 cycles. The deparaffinised samples were first subjected to 25 PCR cycles, then a 50 μl aliquot was taken, increased to 100 μl with a PCR mix (containing 15 pmoles and 20 nmoles of fresh added primers and dNTPs, respectively, and 2.5 U of *Taq* polymerase), and used for a subsequent round of 25 PCR cycles, using a Perkin-Elmer/Cetus thermal cycler. After denaturation at 94°C for 3 min, each cycle consisted of 25 s incubation at 94°C, 1 min annealing at 50°C for H-*ras*1 12, 55°C for K-*ras*2 and N-*ras* 12, 61, and 61°C for H-*ras*1 61, and 1 min elongation at 72°C. In all cases, the PCR

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expected fragments were visualised on ethidium bromide-stained agarose gel.

Oligonucleotide hybridisation

After amplification, 5 μl of the PCR were denaturated in 0.4 M NaOH, 25 mM EDTA at room temperature and spotted on to a nylon membrane (NY13N Schleicher & Schuell). Antisense oligonucleotides for the normal and mutated codons of *ras* genes were also included (4 pmoles of each oligonucleotide). Membranes were neutralised in 0.5 M Tris–HCl pH 7.5, 1.5 M NaCl and baked at 80°C for 1 h. Prehybridisation, hybridisation and washing of filters were performed in a 3 M tetramethylammonium chloride salt solution as described [9].

Direct sequencing

Mutated and control samples were purified on polyacrylamide gel according to Maniatis and colleagues [28]. Template (0.3 pmoles) and one of the amplification primers (15 pmoles) were mixed with the reaction buffer of the Sequenase kit (USB, Cleveland, Ohio, U.S.A.) and NP40 according to Bachmann [29]. After boiling for 3 min, the mix was frozen in liquid nitrogen. Labelling was carried out at 4°C for 3–5 min using 10 μCi of [35 S]dATP and the Sequenase kit. The termination reaction was carried out at 37°C for 3 min using 2 μl of each of four modified termination solutions with a 1 to 1 (8 μM to 8 μM) deoxy- to dideoxy-NTPs ratio. Sequence reactions were electrophoresed on a 8% polyacrylamide, 7 M urea gel.

Analysis of clinical data

For each patient the following characteristics were retrieved from the medical records: age, sex, tumour size, lymph node metastases, distant metastases and recurrence. The association between each of these variables and the presence or absence of RAS mutations was tested by the FREQ procedure of SAS [30]. All *P* values are based on two-tailed Fisher's exact test.

RESULTS

By PCR, we amplified H-*ras*1, K-*ras*2 and N-*ras* genes across codons 12–13 and 61 in 64 DNAs obtained from frozen tumours, as illustrated in Figure 1, and in 37 Bouin-fixed, paraffin-embedded specimens. All amplified DNA samples hybridised to the normal alleles, although with an intersample variation in the hybridisation signal intensity due to a different efficiency of each amplification. Analysis of *ras* genes in the fixed tissues gave results concordant with those obtained from the corresponding frozen tissues (see case 11A in Figure 2).

Thyroid adenomas

None of the 19 FA showed any of the analysed activating *ras* mutations (Table 1).

Follicular carcinomas

Mutated *ras* oncogenes were identified in five out of 21 cases (23.8%), all belonging to the widely invasive variant of FC. Codon 61 of H-*ras*1 was involved three times either at the first base (C→ A transversion) or at the second base (A→ G transition). The remaining two mutated cases showed the same mutation at the second position of codon 61 of N-*ras* gene (A→ G transition) (Tables 1, 2).

Poorly differentiated carcinomas

Thirteen samples of PDCs were collected; among them four were made up of two primary tumours with their respective lymphonodal metastases. PCR analysis showed three mutated samples out of the 11 primary tumours tested (27%). No differences were found between primary tumours and their metastases, both displaying wild type *ras* genes. Among the three mutated tumours, N-*ras* oncogene was altered in two samples, which contained a common alteration at the second base of codon 61 (A→ G transition, samples), and K-*ras*2 gene in the third sample, mutated at the second base of codon 12 (G→ C transversion) (Tables 1, 2).

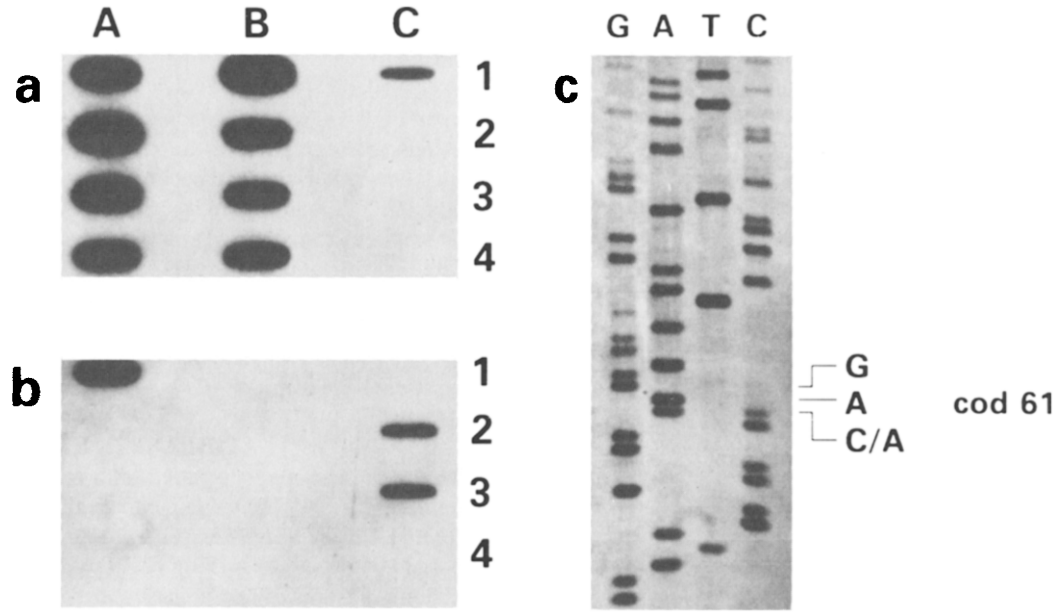

Figure 1. Detection of point mutation at codon 61 of the H-*ras* oncogene in fresh thyroid tumours. (a) Selective oligonucleotide hybridisation with an oligonucleotide specific for wild-type gene. Samples 1-4A and 1-4B are PCR amplified DNAs from frozen thyroid carcinomas; sample 1C is an antisense oligonucleotide for the wild-type HRAS gene codon 61 used as positive control. (b) Selective oligonucleotide hybridisation of the same filter with a mix of two oligonucleotides specific for mutations at the first base of codon 61. Slots 2C and 3C contain antisense oligonucleotides complementary to the probes used. Slot 1A corresponds to sample no. 1, a *ras*-mutated FC. (c) Direct sequencing of the PCR amplified DNA of sample 1, showing a mutation (transversion C→ A, Lys→ Gln) at the first position of codon 61 of H*ras* gene.

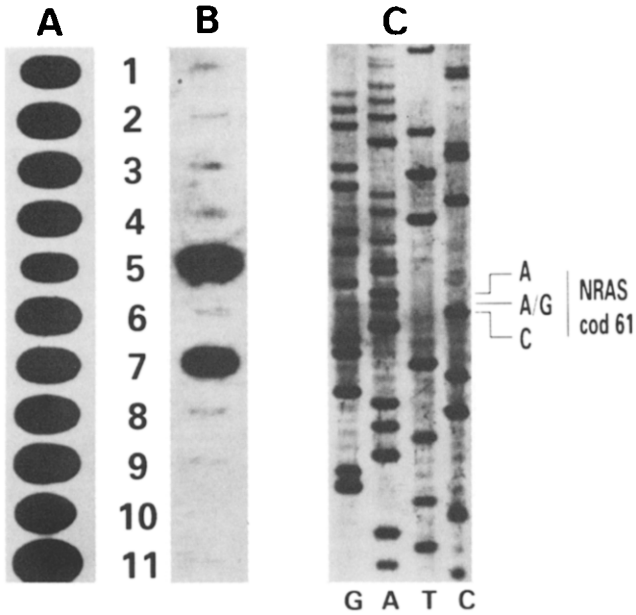

Figure 2. Analysis of N-*ras* gene mutations at codon 61 from PCR-amplified DNAs of paraffin-embedded thyroid tumours. (A) Hybridisation with the wild-type oligonucleotide of N-*ras* gene in and around codon 61. (B) Hybridisation with an oligonucleotide specific for N-*ras* 61-Arg that recognises the mutation altering the normal codon for Gln (CAA) in the codon for Arg (CGA). Samples 11A and 13A (slot 5 and 7, respectively) were mutated. (C) Direct sequence of PCR-amplified DNA of paraffin-embedded sample 11A confirming the mutation at the second base of codon 61 (CAA→ CGA).

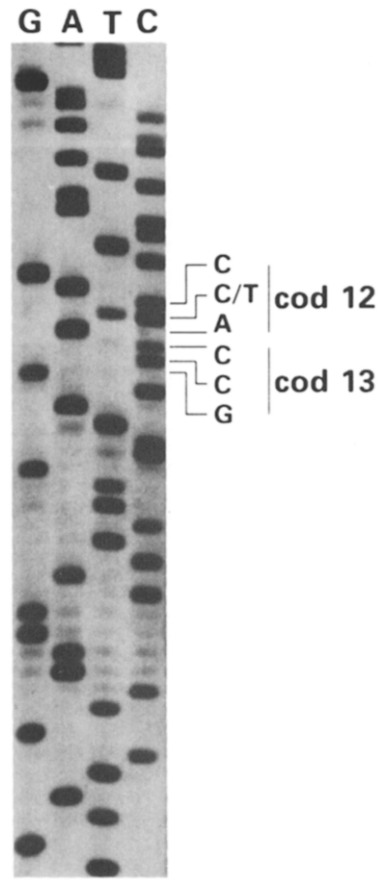

Figure 3. Direct sequence of the antisense strand of PCR-amplified DNA of K-*ras* around codons 12 and 13, in a paraffin-embedded UC (sample 61A). The nucleotide sequence shows mutation at the second base of codon 12 producing a transition GGT→ GAT (ACC→ ATC on the antisense strand) coding for Asp instead of the normal Gly.

Undifferentiated carcinomas

One of five (20%) UC was mutated at the second base of codon 12 of the K-*ras*2 gene (G→ A transition) (Tables 1, 2 and Figure 3).

Papillary carcinomas

We screened for *ras* mutations in 31 primary papillary thyroid tumours and, in 6 cases, the relative nodal metastases. Neither in the primary tumours nor the metastases were *ras* mutations detected. A previous analysis of 51 cases of PC by transfection assay and/or by molecular techniques, including part of those presently analysed, showed that 18 were positive for RET/PTC, eight for TRK and one displayed a mutation at the second position of codon 61 of N-*ras* (C→ A transvertion) (Tables 1, 2). The latter case (sample tir 106) previously classified as PC was excluded from this group, since at revision, it was judged inadequately sampled for a correct histological classification to be made.

Table 1. *Frequency of* ras *gene mutations in thyroid tumours*

Histology	No. of tumours Analysed	Mutated (%)
FA	19	—
FC	21	5 (23.8)
PDC	11	3 (27)
UD	5	1 (20)
PC	31	—

FA, follicular adenoma; FC, follicular carcinoma; PDC, poorly differentiated carcinoma; UD, undifferentiated carcinoma; PC, papillary carcinoma.

ras *mutations and clinical behaviour*

After the completion of the *ras* mutation analysis, we compared our results with the clinical variables. Since, in our study, *ras* mutations were detected only in follicular, poorly differentiated and undifferentiated thyroid carcinomas, we considered only these tumour types (Table 3). A statistically significant correlation was found between the presence of a *ras* mutation in the primary tumour and the appearance of distant metastases ($P = 0.026$). The association became even stronger if only bone metastases were considered ($P = 0.003$) (Table 4).

DISCUSSION

In the present study, we used both frozen and Bouin-fixed, paraffin-embedded tissues to compare the mutation frequency of H-*ras*1, K-*ras*2 and N-*ras* genes in 88 primary and eight nodal metastases of tumours arising from the follicular epithelium of the human thyroid. The accuracy of this evaluation of RAS mutations is supported by the fact that we applied particularly stringent conditions during hybridisation with oligonucleotide probes, and confirmed the positivity of the samples, including those giving a signal higher than background, by direct sequencing. In addition, the inclusion on the slot blot of complementary oligonucleotides specific for each possible mutation ensured that

Table 2. ras *gene family mutations in different types of thyroid tumours*

Histological type	Sample	*ras* codon	Mutation	Pathological stage*
FC	1	H-*ras* 61	CAG → AAG‡	pT4N0M1 (OSS)
	8/11A†	N-*ras* 61	CAA → CGA§	pT3N0M1 (OSS)
	13A	N-*ras* 61	CAA → CGA§	pT3N0M1 (OSS)
	21A	H-*ras* 61	CAG →CGG§	pT2N0M1 (OSS)
	81A	H-*ras* 61	CAG → CGG§	pT3N0M0
PDC	14	K-*ras* 12	GGT → GCT‖	pT4N1aM1R2 (OSS)
	18	N-*ras* 61	CAA → CGA§	rpM1 (OSS)
	Tir103	N-*ras* 61	CAA → CGA§	pT4N1bM1 (OSS)
UC	61A	K-*ras* 12	GGT → GAT¶	T4N1bMX**
Unclassified	Tir106	N-*ras* 61	CAA → AAA‡	Lost*

FC, collicular carcinoma; PDC, poorly differentiated carcinoma; UC, undifferentiated carcinoma. *TNM system [36]. †The tumour was analysed from both frozen (8) and Bouin-fixed paraffin-embedded (11A) material with the same result. Amino acid substitution: ‡Gln→ Lys; §Gln→ Arg; ‖Gln→ Ala; ¶Gly→ Asp. **Incisional biopsy.

the sensitivity of hybridisation and washing conditions were adequate to allow each probe to anneal with its complementary sequence. This is important since false results could significantly affect the conclusion of such an analysis. It is also possible that other factors, such as the dietary iodine intake [14] or the diagnostic criteria applied for the histological classification, could have strongly influenced the evaluation of the frequency of *ras* activation in samples collected from separate geographical areas and analysed in different laboratories [11-14].

Our analysis of human thyroid tumours, all collected in our Institute, confirmed the occurrence of mutations in all three *ras* genes as already indicated by other reports [11, 12, 14]. Irrespective of the affected *ras* gene, mutations were more frequently transitions than transversions (seven versus three). The most commonly detected amino acid substitution was that of glutamine to arginine (five of 10 mutated cases) arising from an A to G transition at codon 61 of H-*ras*1 and N-*ras* genes, in agreement with the results of Lemoine and colleagues [11]. In our study, mutations involving K-*ras* were located exclusively at the codon 12, while both H-*ras*1 and N-*ras* showed mutations only at codon 61.

As mentioned above, there are discrepancies between the frequency of mutations of *ras* genes in different thyroid tumour types in different studies. In our study, the *ras* gene family does not seem to play a major role in the pathogenesis of PC, at variance with previous reports showing a rate of *ras* mutations varying from high to intermediary or low frequencies of *ras* activation in this tumour type [12, 13, 15, 31]. So far, we have not identified well differentiated PCs with *ras* mutations following a reclassification of our cases according to updated criteria [20–24]. The relevance of the categorisation criteria is strengthened by our previously reported transfection analysis of 51 samples of PC, including 24 of the 31 cases reported here, where we found activation of tyrosine kinase oncogenes, RET/PTC and TRK, in 35 and 15% of the cases, respectively ([17, 32] and unpublished results). *ras* mutations were reported in two samples, but a subsequent blind histological revision redefined the first case (Tir 103) as PDC, insular carcinoma variant, whereas the second sample (Tir 106), obtained by incisional biopsy in a patient with extensive neck disease, was inadequate for a correct classification. Furthermore, Shi and colleagues [14], analysing a series of PC derived from two different geographical areas, did not find any mutations in the 22 tested cases. The present result suggests that *ras* gene mutations and activation of tyrosine kinase genes might behave as mutually exclusive oncogenic pathways in well differentiated thyroid carcinomas.

In the case of follicular tumours, a significant frequency of mutations of *ras* oncogenes has been detected by several laboratories in both FA and FC as well as in the UC. The cumulative results of different studies reported by Wright and colleagues [15] found 52% of FC and 55% of UC mutated. Moreover, Shi and colleagues, by comparing the frequency of *ras* mutation in FCs derived from iodine deficient areas with those belonging to areas with high iodine intake, found a higher rate of *ras* mutations in the former samples (50 versus 10%), suggesting that this element can affect the frequency of *ras* activation [14]. Our results showed a rate of mutations lower than those reported by Wright and colleagues [15] and by Shi and colleagues [14] for the FC and UC. In fact, we detected *ras* mutation in 23.8% (5/21) of the FCs and 20% (1/5) of the UCs. We also identified the same type of genetic change in 27% (3/11) of a more recently defined group of thyroid carcinomas (PDC), which may represent an intermediate form in the transition phase from well differentiated to undifferentiated carcinomas.

Unlike FCs, in none of the 19 examined FAs were RAS mutations detected. This result is at variance with reports from other laboratories, in which a high percentage were positive, suggesting that *ras* plays a role as an initiator of epithelial thyroid tumorigenesis [11–14, 33]. In our cases, *ras* oncogene mutations were confined to FC (all invasive variant), PDC and UC. This observation could imply that these oncogenes play a role in progression rather than in the initiating events of thyroid tumour development as suggested by different groups [11–14, 33]. However, as previously discussed, genetic and/or environmental factors or sample bias could account for these discrepancies.

The clinical characteristics of the FCs, that typically show haematogenous spreading, and the invariably fatal behaviour of PDC and UC, both suggest that *ras* activation could represent an indicator of unfavourable prognosis. The significant association reported here, between the presence of *ras* mutations and the occurrence of bone metastases ($P = 0.003$), is in keeping with this possibility, although a larger number of observations are required to confirm this result. Furthermore, additional studies are also necessary to determine the role and the relationship

Table 3. Histological and clinical parameters of thyroid tumours included in the statistical analysis for correlation with ras *mutation*

Sample	Type	Histology Variant†	*ras* mutation	Pathological stage (pTNM)* At presentation	Recurrence
2A	FC	Minimally invasive	−	pT2N0M0	—
4A		Minimally invasive	−	pT3N0M0	—
12A		Minimally invasive	−	pT2N0M0	—
76A		Minimally invasive	−	pT3N0M1 (PUL)	—
79A		Minimally invasive	−	pT3N0M0	—
1	FC	Invasive	+	pT4N0M1 (OSS)	—
5		Invasive	−	pT3N0M1 (OSS)	—
6		Invasive	−	pT2N0M1 (OSS)	—
7		Invasive	−	pT4N0M1 (OSS)	—
8		Invasive	+	pT3N0M1 (OSS)	—
9		Invasive	−	pT2N0M0	—
5A		Invasive	−	pT3N0M0	—
13A		Invasive	+	pT3N0M1 (OSS)	—
14A		Invasive	−	pT3N0M0	—
15A		Invasive	−	pT4N0M0	—
17A		Invasive	−	pT3N0M0	—
18A		Invasive	−	n.a.	n.a.
21A		Invasive	+	pT2N0M1 (OSS)	—
72A		Invasive	−	pT2N1aM0	—
78A		Invasive	−	**	—
81A		Invasive	+	pT3N0M0	—
12	PDC	Insular	−	pT3N0M1 (PUL, OSS)	rpT4N1aM0
16A		Insular	−	Insular carcinoma,	rpM1 (OSS)
18		Insular	+	elsewhere	rpM1 (PUL, OSS)
58A		Insular	−	Insular carcinoma,	—
Tir 103		Insular	+	elsewhere pT4N1aM0 pT4N1bM1 (OSS)	
14	PDC	Sakamoto	+	pT4N1aM1R2 (OSS)	—
37A		Sakamoto	−	pT4N1aM1 (PUL, HEP)	—
17	PDC	Oxyphilic cell	−	pT4N1aM1 (PUL)	—
55A		Oxyphilic cell	−	pT4NOM1 (OSS)	—
35	PDC	Tall cell	−	pT4N1aM0	—
10	PDC	Trabecular	−	pT4N1aM0	rpM1 (OSS, SKIN)
56A	UC	Fibrosarcoma-like	−	pT4N0M0	—
62A		Fibrosacoma-like	−	pT4N1bM0	—
13	UC	Giant cell	−	pT4N1aM1 (HEP, OTH‡)	—
61A		Giant cell	+	T4N1bMX§	—
15	UC	Squamous cell carcinoma	−	pT4N0M1 (SKIN)	—

FC, follicular carcinoma; PDC, poorly differentiated carcinoma; UC, undifferentiated carcinoma; n.a., not available; PUL, pulmonary; OSS, bone; HEP, liver; OTH, other. *TNM system [36]. †For more details, see text. ‡Adrenal gland. §Incisional biopsy. **B (Synchronous squamous cell carcinoma of the oesophagus).

between *ras* activation and p53 mutations that have been associated with PDC and UC [34, 35].

In conclusion, the results of this study indicate that, (i) mutations in all three *ras* oncogenes are significantly associated with widely invasive FC and with less well differentiated carcinomas, PDC and UC, which could be derived from well differentiated carcinomas by a progressive differentiation loss; (ii) *ras* mutations and the activation of tyrosine kinase oncogenes are mutually exclusive in well differentiated PCs and FCs; and (iii) *ras* activation could be significantly associated with the appearance of haematogenous (particularly bone) metastases.

Table 4. Distribution of ras *mutations and distant metastases in the group of thyroid carcinomas including FC, PDC and UC*

ras	Distant metastases* Absence	Distant metastases* Presence	Bone metastases Absence	Bone metastases Presence
Wild type	15	12	21	6
Mutated	1	8†	1	7‡

*Skin, lung, bones, liver, adrenal gland or unknown site.† $P = 0.026$ in Fisher's exact test.‡ $P = 0.003$ in Fisher's exact test.

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