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Ras mutations are uncommon in sporadic thyroid cancer in children and young adults

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ABSTRACT. Mutations in the ras genes (H-ras, K-ras, and N-ras) occur in 10-15% of all human cancers, and commonly arise from single base substitutions at codons 12, 13, or 61. Although ras mutations have been found in adult thyroid cancers, they were absent from the two studies which examined childhood thyroid cancers. Both studies included only children with radiation induced thyroid cancer, and it remains unclear if ras mutations occur in children without radiation exposure. To answer this question, we examined archival tissue blocks from 31 children with papillary thyroid cancer (PTC) 4 with follicular thyroid cancer (FTC), 2 with medullary thyroid cancer (MTC), and 1 with lymphoma (LYM). Only 1 patient with PTC had previous radiation exposure. Genomic DNA was extracted and used for PCR amplification of the ras genes. The PCR products were analyzed by oligospecific hybridization for mutations at codons 12, 13, and 61. Two of the PTCs (6.5%) contained ras mutations. Both patients had class II disease and no history of previous radiation exposure. One patient subsequently developed bone and lung metastases. The patient with lymphoma also had a ras mutation (N-61), but ras mutations were absent from all FTC and MTC. These results suggest that ras mutations are uncommon in spontaneous childhood thyroid cancer, but occur with a frequency similar to that found in previous reports of adult differentiated thyroid cancers. The number of subjects was too small to determine if ras mutations are more common in patients with aggressive papillary thyroid cancer.
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INTRODUCTION

Mutations in the transforming ras genes are some of the most common mutations identified in human cancers. These activating mutations occur with an estimated frequency of 10-15% and commonly arise from single base substitutions which affect either the GTP binding domain (codons 12, 13) or the GTPase domain (codon 61) of the ras protein (1-4).
Adult thyroid tumors frequently contain ras mutations (5).However, mutations of the ras oncogenes are found in both benign and malignant thyroid tumors, suggesting these mutations may occur early in the process of thyroid cell transformation (4). In addition, the frequency of ras mutations has varied greatly depending on the study (6). In papillary thyroid carcinomas (PTC), the frequency of ras mutations has ranged from 6 to 62% compared to 0 to 100% in follicular thyroid carcinomas (FTC) and 33 to 46% in follicular adenomas (5, 7-17). Most of these studies were unable to show any correlation between the presence of activating ras mutations and either a specific histological type or biological behavior of the tumor (9). However, Hara *et al.* did show that N-ras mutations were an independent risk factor for aggressive behavior in PTC (7).
Only two studies have evaluated childhood thyroid cancers for the presence or absence of ras mutations. Of note, all the children in these studies had been exposed to radiation from the Chernobyl nuclear accident. Neither the study by Nikiforov *et al.* which investigated 33 childhood PTCs, nor that of Suchy *et al*. which analyzed 34 PTCs, found any mutations in the critical ras codons in either the H-, K-, or N-ras genes (10, 11).

Key-words: Ras, thyroid, cancer, childhood.

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To our knowledge no studies have yet explored the role of ras oncogene mutations in the development of spontaneous differentiated thyroid cancer (DTC) in children who did not have previous radiation exposure. Several observations suggest that spontaneous DTC in children and adolescents might be different than in adults. DTC is less common in children, and only 10% of new cases occur in the pediatric age group (18-21) . The majority of children with PTC present with local metastasis, but very few die of disease or develop anaplastic thyroid cancer. Furthermore, mutations in the alpha subunit of the GTP binding protein complex (GSα), which are common in adult PTC, are rarely found in children with PTC (22). The current study is the first to determine the presence of H-, K-, and N-ras mutations in spontaneous childhood thyroid cancer and the first to relate the presence of ras mutations to clinical outcome in children. The major findings of this study are that ras mutations are uncommon in spontaneous childhood thyroid cancers (6.5% of PTC).

Because of this low incidence, the prognostic significance of ras mutations is still uncertain in this age group.

MATERIALS AND METHODS

This study received prior approval by the Human Use Committee and funding support (WU# 6414) from the Department of Clinical Investigation, Walter Reed Army Medical Center, Washington, DC.

Materials

The centralized tumor registry for the Department of Defense (ACTUR) was searched to identify patients who were diagnosed with thyroid cancer prior to 21 years of age. The clinical data for a portion of these patients (137 with PTC and 33 with FTC) has been previously published (21).

Histology

Original paraffin embedded tissue blocks were obtained from 38 of these patients and used to prepare routine histology slides which confirmed the pathological diagnosis. There were a total of 31 patients (81.6%) with PTC, two (10.5%) with FTC, two (5.3%) with medullary thyroid cancer (MTC), and one (2.6%) with intrathyroidal lymphoma (LYM).

Clinical data

The clinical details of the subjects are shown in Table 1. Only 1 patient had a clearly documented history of previous radiation exposure. This patient (#11) was treated with external beam radiation at the age of 8 years for treatment of Hodgkin's lymphoma. His thyroid cancer was first detected 11 years later. One additional patient (#28) had lived near a nuclear power plant from the age of 7 to 12 years, but had no known exposure to radiation. A thorough review of the medical records revealed nothing to suggest exposure to ionizing radiation in any other patient.

Papillary thyroid cancer

Papillary thyroid cancers from 31 patients (23 females and 8 males) were examined. The details of tumor class, treatment, adjunctive therapy, and recurrence are shown in Table 1. The sex distribution is consistent with many previous studies in which the overall ratio of females to males is approximately 2:1 (21, 23). The median age at diagnosis was 17 years (range 6-21 years) and 45.1% <17 years of age, 12.9% were between the ages of 17 and 19, and 42.0% were ≥19 years of age. The majority of patients (87.9%) were defined as having DeGroot class I or class II disease (24). This is consistent with previous studies where the majority of young patients have either class I or class II PTC (24). The median postoperative follow-up was 63 months (range 0 to 246 months), with 55% having follow up of ≥5 years. At last follow-up, 22 patients (71%) were alive without evidence of disease. This included 4 patients who are currently disease free, but who had developed recurrent disease and were successfully treated. One patient (#27, 3.2%) was alive with persistent disease; 3 patients (9.7%) were alive with recurrent disease, 2 patients (6.5%) were known to be alive but their disease status was uncertain, and 3 patients (9.7%) had no long-term follow-up data available.

Follicular thyroid cancer

Follicular thyroid cancers from 4 patients (3 females and 1 male) were examined. The details of tumor class, treatment, adjunctive therapy, and recurrence are shown in Table 1. The median age at diagnosis was 18 years (range 13-20 years). Of these patients, 1 was <17 years of age, 1 was between the ages of 17 and 19, and 2 were ≥19 years of age. At last follow-up all 4 patients (100%) were alive without evidence of disease.

Others

MTCs from 2 patients (1 female patient aged 17 years and 1 male patient aged 21 years) were also examined. At last follow-up 1 patient (#36) had died of MTC and the second was alive but the disease status is unknown. The single female patient with intrathyroidal lymphoma was diagnosed at age 21 years and, at last follow-up, was alive with persistent disease.

Table 1 - *Demographics of patients with thyroid cancer.*

Case	Age/sex	Radiation exposure	Extent of surgery	131-Iodine ablation	Recurrence	Focality	Follow-up (months)	Clinical status
Patients with PTC								
Class I								
1	6/F	N	Total	N/A	N	Unifocal	0	N/A
2	11/F	N	Subtotal	Y	N	Unifocal	18	NED
3	14/M	N	Total	Y	Y/11 m	Unifocal	63	NED
4	14/F	N	Total	N/A	N	Unifocal	246	NED
5	14/M	N	Total	N	N	Multifocal	10	NED
6	15/F	N	Total	Y	N	Unifocal	50	NED
7	17/F	N	Total	Y	N	Unifocal	30	NED
8	17/F	N	Total	Y	N	Unifocal	50	NED
9	18/F	N	Total	Y	Y/8 m	Unifocal	100	NED
10	19/F	N	Subtotal	Y	N	Unifocal	106	NED
11	19/M	Y	Subtotal	N/A	N	Unifocal	77	AD
12	20/M	N	Total	Y	N	Multifocal	24	NED
13	20/M	N	Subtotal	N	N	Unifocal	118	NED
14	21/F	N	Total	N/A	N	Unifocal	68	NED
15	21/F	N	Total	N/A	N	Multifocal	18	NED
Class II								
16	13/F	N	Subtotal	N/A	N	Multifocal	0	N/A
17	13/M	N	Subtotal	Y	Y/159 m	Multifocal	161	AD
18	14/F	N	Total	Y	Y/67 m	Multifocal	86	NED
19	15/F	N	Total	Y	N	Multifocal	27	NED
20	15/F	N	Total	Y	N	Multifocal	85	NED
21	17/F	N	Total	Y	N	Multifocal	116	NED
22	19/F	N	Total	N/A	N	Unifocal	156	Alive, N/A
23	19/F	N	Total	Y	N	Multifocal	37	NED
24	20/F	N	Total	Y	N	Unifocal	101	NED
25	20/F	N	Total	Y	N	Unifocal	92	NED
26	21/F	N	Total	N/A	N	Multifocal	22	Alive, N/A
Class III								
27	10/M	N	Subtotal	Y	N	Unifocal	27	AD
28	15/M	N	Total	Y	Y/40 m	N/A	60	AD
29	19/F	N	Total	Y	Y/17 m	Multifocal	86	AD
Class IV								
30	6/F	N	Near total	Y	Y/6 m	Multifocal	169	AD
31	20/F	N	Total	Y	N	Multifocal	63	NED
Patients with FTC								
32	13/M	N	Partial	Y	N	Unifocal	168	NED
33	17/F	N	Total	Y	N	Unifocal	43	NED
34	19/F	N	Total	N/A	N	Unifocal	18	NED
35	20/F	N	Total	Y	N	Unifocal	34	NED
Patient with MTC								
36	17/F	N	Total	N	Y/120 m	N/A	180	DTC
37	21/M	N	Total	N/A	N	Multifocal	59	Alive, N/A
Patients with LYM								
38	21/F	N	Partial	N	N	N/A	57	AD

PTC=papillary thyroid cancer, FTC= follicular thyroid cancer, MTC=medullary thyroid cancer, LYM=lymphoma; *Class=disease class according to DeGroot *et al*. (24); Surgery=extent of initial operative procedure; 131 Iodine ablation=received I^{131} therapy; Recurrence=recurrence and/months after initial therapy when recurrence was first detected; Focality=multifocal *vs* unifocal lesions at diagnosis; Follow-up=length of follow-up in months; and Clinical status=N/A=not available, AD=alive with disease, NED=no evidence of disease, DTC=died of thyroid cancer.

DNA extraction

The 5 sections immediately adjacent to the diagnostic slides (5 µm/section) from each block were pooled and used to prepare genomic DNA extracts using a modification of a previously described method (25). Paraffin embedded tissue was deparaffinized with xylene and rehydrated through a series of graded alcohol solutions and nuclease free diethylpyrocarbonate (DEPC) treated water. The tissue was digested with proteinase K (48 hours, 52 C) and the nucleic acids were isolated using phenol and chloroform/isoamyl alcohol (49:1, v:v). Genomic DNA was extracted from the organic phase using 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 1% SDS, pH 12.15 (volume of 500 µl) and 80 µl NaOH (10M). The DNA was precipitated and washed in cold ethanol, suspended in 1X Tris-EDTA (10 mMTris-HCl, 1 mM EDTA), pH 7.4, and stored at 4 C.

Polymerase chain reaction

One hundred nanograms of genomic DNA served as substrate for the PCR reaction. Final reaction conditions were as follows: 1X Buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5-3 mM $MgCl_2$ 200 µM each dNTP, 200 µM of each outside primer, 0.03 U/µ AmpliTaq Gold (Perkin Elmer). The cycling conditions included an initial phase of 5 minutes at 95 C to activate the AmpliTaq Gold which was followed by a single cycle of melting at 95 C (30 seconds), annealing at 57 C (30 seconds), and extension at 74 C (30 seconds). For the next 9 cycles, the annealing temperature was lowered 1 C/cycle (from 57 C to 48 C) for a total of 10 cycles (touchdown PCR). At that point, an annealing temperature of 47 C was used for the next 25 cycles. A final 74 C extension phase for 7 minutes completed the PCR thermocycling.

Ten to twenty percent of the first round PCR product served as template for the nested PCR primers (Table 2). PCR reaction conditions and thermocycling parameters were identical to those described for first round PCR above. PCR products were analyzed by electrophoresis through 1.8% agarose gels followed by ethidium bromide staining to ensure amplification of the appropriately sized product.

Allele specific hybridization

The amplified DNA from the nested PCR reaction was then utilized for allele-specific oligonucleotide hybridization as previously described (10). In brief, 20 microliters of the nested PCR product was denatured (0.4M NaOH, 24 mM EDTA, 95 C 2 minutes), neutralized with cold 1M TRIS and dot blotted onto Amersham Hybond-N™ nylon membranes using the Schleicher & Schuell Minifold™ manifold. The filters were dried and cross-linked with ultraviolet light. Specific wild type and mutant ras oligonucleotide probes (Table 3) were [^{32}p] 5′ end labeled with T4 polynucleotide kinase (CLONTECH, Palo Alto, CA) according to the manufacturers suggested instructions. Filters were hybridized overnight at 37 C with a combination of either the wild type probe or mutant probe panels. The membrane was then rinsed in 6X SSC for 1 minute, followed by 6X SSC, 0.1% SDS for 30 minutes at the calculated (Tm+1C) for each probe. Tm was calculated by the formula: Tm=69.3+0.41 (G+C)%. Oligospecific hybridization was then detected by autoradiography.

Oligospecific hybridization assay validation

DNA was recovered from three K-ras mutant cell lines [CALU-1 cell line, NCI-H-1155 cell line, and the SW - 480 cell line (ATCC)] and an additional 8 adult PTC

Table 2 - *Primer sets for nested PCR.*

	Inside primer pair		Outside primer pair	
H-ras 12/13	ATG ACG GAA TAT AAG CTG GT CGC CAG GCT CAC CTC TAT A	122bp	TGA GGA GCGATG ACG GAA TAT AGG ACG TCT CCC CAT CAA TGA CCA C	427bp
H-ras 61	GGT GGT CAT TGA TGG GGA G AGG AAG CCC TCC CCG	108bp	CAT TTT GTGGAC GAA TAC GAC CC GGT TCA CCT GTA CTG GTG GAT GTC	486bp
K-ras 12/13	ATG ACT GAA TAT AAA CTT GT CTC TAT TCT TGC ATC ATA TT	111bp	GCC TGC TGA AAA TGA CTG CAA AGA ATG GTC CTG CAC	170bp
K-ras 61	GCA ACT ACT AAT TGA TGG AG AGA AAG CCC TCC CCA G	111bp TGC	CAG ACT GTG TTT CTC CCT TCT CAG G TCT AAT CCCCCA AGA ACT TC	372bp
N-ras 12/13	ATC ACT GAG TAC AAA CTG GT CTC TAT GGT GGG ATC ATA TT	111bp	GTT CTT GCT GGT GGT GTG AAA TG TCA CCT CTA TGG TGG GAT C	133bp
N-ras 61	CAA GTG GTT ATA GAT GGT GA AGG AAG CCT TCG CCT G	110bp	CAA GTG GTT ATA GAT GGT GA ATG ACT CCT AGT ACC TGT AGA GG	178bp

All primer sets are listed 5′→3′. The upper sequence in each pair is the upstream primer and the lower sequence is the dowstream primer.

Table 3 - *Allele specific hybridization sequences.*

Gene/ codon	Probe sequence	Amino acid	Gene/ codon	Probe sequence	Amino acid
Ha-ras/12	GGC GCC GGC GGT GTG GGC AA	Gly (wt)	Ki-ras/12	GGA GCT GGT GGC GTA GGC AA	Gly (wt)
	AGC	Ser		AGT	Ser
	TGC	Cys		TGT	Cys
	CGC	Arg		CGT	Arg
	GAC	Asp		GAT	Asp
	GTC	Val		GCT	Ala
	GCC	Ala		GTT	Val
Ha-ras/13	GGC GCC GGC GGT GTG GGC AA	Gly (wt)	Ki-ras/13	GGS GCT GGT GGC CTA GGC AA	Gly (wt)
	AGT	Ser		AGC	Ser
	TGT	Cys		TGC	Cys
	CGT	Arg		CGC	Arg
	GAT	Asp		GAC	Asp
	GCT	Ala		GTC	Val
	GTT	Val		GCC	Ala
Ha-ras/61	ACC GCC GGC CAG GAG GAG TA	Gln (wt)	Ki-ras/61	ACA GCA GGT CAA GAG GAG TA	Gln (wt)
	GAG	Glu		GAA	Glu
	AAG	Lys		AAA	Lys
	CGG	Arg		CGA	Arg
	CTG	Leu		CTA	Leu
	CCG	Pro		CCA	Pro
	CAT	His #1		CAT	His #1
	CAC	His #2		CAC	His #2
N-ras/12	GGA GCA GGT GGT GTT GGG AA	Gly (wt)			
	AGT	Ser			
	TGT	Cys			
	CGT	Arg			
	GAT	Asp			
	GCT	Ala			
	GTT	Val			
N-ras/13	GGA GCA GGT GGT GTT GGG AA	Gly (wt)			
	AGT	Ser			
	TGT	Cys			
	CGT	Arg			
	GAT	Asp			
	GCT	Ala			
	GTT	Val			
N-ras/61	ACA GCT GGA CAA GAA GAG TA	Gln (wt)			
	GAA	Glu			
	AAA	Lys			
	CGA	Arg			
	CTA	Leu			
	CCA	Pro			
	CAT	His #1			
	CAC	His #2			

Wt=wild type; all probes are listed 5'→3'; standard three letter abbreviations are used for the amino acids.

samples. PCR was performed as outlined above. Oligospecific hybridization was performed for all possible K-ras mutations which detected 1 tumor sample to have a K-ras 61 mutation as well as all the anticipated mutations in all three positive controls. All other samples were negative for ras mutations. Direct sequencing of all 8 adult PTC and the positive controls confirmed the oligospecific hybridization. The K-ras 61 mutation was shown to have the mutant sequence CAA→CAT (resulting in a Gln to His amino acid substitution). The positive controls were also shown to have the mutant sequence: CALU-1 (K-ras 12: GGT→TGT) (Fig. 1), NCI-H-1155 (K-ras 61: CAA→CAT), and SW-480 (K-ras 12: GGT→GTT). All other samples contained only the wild type ras.

Single strand conformational polymorphism analysis

The cell line positives, the tumor sample with the K-ras 61 mutation and 3 of the 7 remaining adult PTCs were examined by single strand conformational polymor-

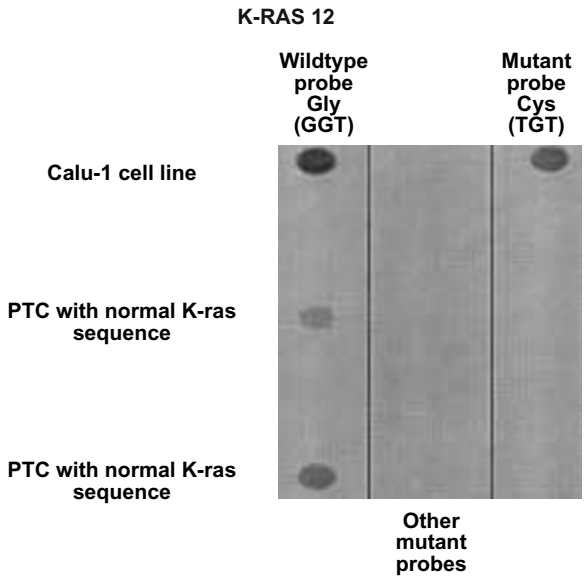

Fig. 1 - *Oligonucleotide probe specificity is shown for K-ras 12. A nested PCR strategy was used to amplify the K-ras gene in the codon 12 region using DNA recovered from the Calu-1 cell line (known K-ras positive mutation), and 2 other thyroid cancer specimens which had previously been shown by DNA sequencing to contain the wildtype (normal) K-ras sequences. The PCR products were dot blotted onto a single membrane and probed sequentially with wild type Gly probe (Lane 1), a panel of all other mutant probes (Lane 2), and the mutant Cys probe (Lane 3). As expected, wild type K-ras 12 was detected in each sample. Only the known positive Calu-1 cell line demonstrated the presence of the G→T mutation, resulting in a Gly to Cys substitution.*

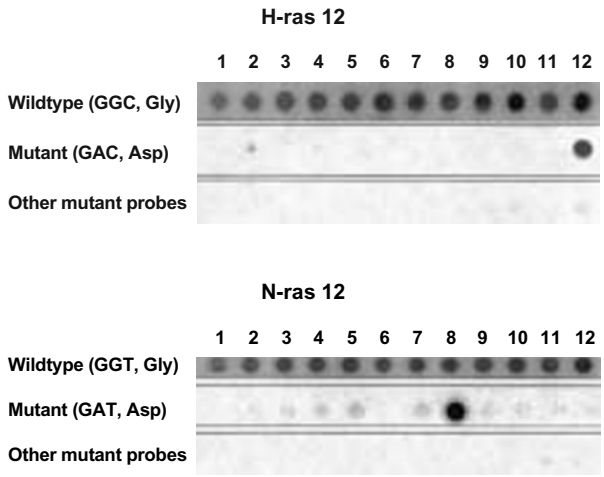

Fig. 2 - *Allele specific oligonucleotide hybridization for selected patients with H-ras 12 and N-ras 12 mutations. The upper panel shows the results of hybridization of the amplified PCR products with either an oligonucleotide probe specific for wild type H-ras 12 or mutant probe panels specific for H-ras 12. Wild type H-ras 12 was detected in each sample. A single patient (#21, Lane12) was shown to have a single base mutation (GGC→GAC) which resulted in a Gly to Asp amino acid substitution. No mutations were found using the other mutant probes.*
The lower panel shows the results of hybridization of the amplified PCR products with either an oligonucleotide probe specific for wild type N-ras 12 or mutant probe panels specific for N-ras 12. Wild type N-ras 12 was detected in each sample. A single patient (#17, Lane 8) was shown to have a single base mutation (GGT→GAT) which resulted in a Gly to Asp amino acid substitution. No mutations were found using the other mutant probes.

phism analysis (SSCP). The internal PCR primer pair outlined above was flourescein labeled. The forward primer was 5′ labeled with FAM and the reverse primer was 5′ labeled with JOE (ADI). Standard PCR was performed as described above. The labeled PCR products were combined with ROX 2500 internal size standards and subjected to SSCP analysis utilizing a Proto-Gel (2.5% glycerol/1X TBE) at 20 C for 7 hours on the PE/ABI 373 DNA sequencer. SSCP confirmed the oligospecific hybridization results in each case.

Statistical analysis

Statistical analysis was performed using SPSS for Windows 95 (Version 7.5, SPSS Inc., Chicago, IL). The Chi-square and Fisher's exact tests were used as indicated for analysis of nominal variables.

RESULTS

RAS

Wild type ras was successfully amplified in all samples and taken as evidence for the presence of intact DNA isolation. Positive controls were consistently detected and negative controls were uniformly negative (Fig. 1). Mutations in the ras oncogenes were identified in two of the 31 (6.5%) patients with PTC (Fig. 2) The first patient (#21, Lane 12) presented with class II disease and was shown to have a GGC→GAC mutation in codon 12 of the H-ras oncogene resulting in a glycine (Gly) to aspartic acid (Asp) amino acid substitution. The tumor had a diameter of 2 cm and was multifocal with spread to ipsilateral lymph nodes. The patient was followed for a total of 116 months, during which there were no signs of recurrence. The second patient (#17, Lane 8) also had class II disease at diagnosis and was shown to have a GGT→GAT mutation in codon 12 of the N-ras gene resulting in a Gly to Asp amino acid substitution. The tumor had a diameter of 4.2 cm, but was multifocal with spread to the ipsilateral lymph nodes. In contrast to the first patient, this patient developed distant recurrence to both the lung and bones at approximately 159 months after initial diagnosis.
In our previous study larger tumor size, multifocal disease and more extensive disease at diagnosis

were shown to predict recurrence for children with PTC (21). In the current study, there was no significant association between ras mutations and either tumor size, focality, direct extension, tumor class, metastasis, or recurrence (p=ns for all comparisons, Chi-Square).

The patient with the intrathyroidal lymphoma (#38) had a CAA→AAA mutation in codon 61 of the N-ras oncogene resulting in a glutamine (Gln) to lysine (Lys) amino acid substitution (data not shown). None of the patients with ras mutations had a history of prior radiation exposure. None of the FTC or MTC were found to have a mutation in any of the three ras oncogenes.

DISCUSSION

Although less common than in adults, thyroid cancer is assuming greater importance in children because the frequency has dramatically increased in regions exposed to radioactive fallout surrounding the Chernobyl nuclear accident. Of major world-wide concern are suggestions that the incidence has also increased in regions not exposed to Chernobyl including Sweden, England, and Wales (26).

Children with DTC are commonly thought to have a good prognosis despite the fact that almost half present with metastatic disease at the time of diagnosis (22) . This is based on several studies which have shown that the prognosis for DTC is favorable in patients under 40 years of age (20, 27-30). Our recent retrospective study has confirmed that disease specific mortality is low in children, but has shown that recurrence is common and develops in approximately 19.7% of patients with PTC and 15.2% for patients with FTC (21). Four clinical parameters were found to be useful in predicting recurrence in children and young patients including: multifocal disease, large tumor size, palpable cervical lymphadenopathy at diagnosis, and metastatic disease at diagnosis (21).

Ras mutations occur in adult PTC with variable frequency (6-62%), however, two previous studies failed to identify any ras mutations in childhood thyroid cancers (5-7, 9-14, 17, 31-34). Of note, all the children had been exposed to radioactive fallout from the Chernobyl nuclear accident (10, 11). For that reason, the present study was designed to determine if: 1) mutations in the ras oncogenes occur in spontaneous thyroid cancers in children who were not previously exposed to radiation, and 2) if the presence of ras mutations might be useful in predicting the clinical behavior of thyroid cancer in individual children.

In the current study, ras mutations were absent from all 15 children with PTC confined to the thyroid gland (class I disease). In contrast, 2 out of 16 patients with extrathyroidal PTC (class II, III, or IV disease) were found to contain ras mutations. Neither of these two patients had previous radiation exposure.

The overall frequency of ras mutations was 6.5% in patients with PTC and 12.5% in patients with extrathyroidal PTC. This frequency is similar to those reported by Karga *et al.* and Goretzki *et al.* in their studies of adult PTC (32, 33). Karga's group looked at the frequency of ras mutations in 15 PTCs for which there was no history of prior radiation exposure. Of these 6.7% (1/15) of the PTCs contained a ras mutation [H- 12 (Gly to Ser)]. Goretzki, *et al*. examined 23 PTCs and 9 FTCs for which radiation exposure history was not reported. A total of 12% (9/32) contained ras mutations, all of which were located on the Nras gene at codons 12, 13 or 61.

Our results are very dissimilar from those reported by Wright *et al.* and Du Villard *et al.* (13, 14). Wright found that 50% of the study patients had ras mutations; however, each patient had a history of prior radiation exposure. In the study by Du Villard, only 10% of the patients had a history of prior radiation exposure, but the incidence of ras mutations was 45%. Goretzki *et al.* previously hypothesized that the frequency of ras mutations may be higher in elderly patients (33). The ages of the patients with PTC in the study by Du Villard *et al*. were not specified. It is possible that our study included only younger patients. This could be one possible explanation for the difference in reported frequencies between our study and that by Du Villard *et al.*

Two previous studies have examined a combined total of 67 childhood papillary thyroid cancers and found no ras mutations (CI 0% to 4.5%) (10, 11). All the children in these studies were exposed to radiation from the Chernobyl nuclear accident. There is a suggestion (p=0.096, Fisher's exact test) that the incidence of ras mutations in our study (6.5%, CI 1.2% to 23.5%) might be higher. This suggests the possibility that ras mutations could be more frequent in spontaneous childhood thyroid cancer compared to radiation induced thyroid cancer. Further study will be necessary to answer this question.

In our current study, both of the patients with ras mutations had class II disease at diagnosis. This suggests that ras mutations may be more common with extrathyroidal PTC (p=0.48, Fisher's exact test, 2/16 *vs* 2/31); however, no ras mutations were found in cases with more disseminated disease (class III and IV disease). In adults, ras mutations have been shown to occur in the most histologically and clinically aggressive PTC (67).

In our previous study, larger tumor size, multifocal

disease, and more extensive disease at diagnosis were shown to predict recurrence (21). In the current study ras mutations were not associated with tumor size, focality, direct invasion, tumor class, metastasis, or recurrence (*p*=ns for all comparisons, Chi-Square).

Our study found no ras mutations in any of the four patients with FTC. This is in direct contrast to a study of post-Chernobyl tumors where the single FTC examined revealed a mutation in the N-ras oncogene at codon 61 resulting in a Gln to Lys substitution (35). This suggests a possible role for ras mutations in the pathogenesis of radiation induced FTC, but not in children with spontaneous FTC.

The third patient in our study with a ras mutation had intrathyroidal lymphoma. Sakalidou *et al.* previously examined pan-ras p21 protein expression in childhood lymphomas and found evidence of ras mutations (1). In 39 cases of childhood lymphoma, pan-ras p21 protein was expressed in very few cells; however, 48% of Hodgkin's disease cases showed low level staining in 2-10% of cells. In patients with non-Hodgkin's lymphoma less than 1% of cells stained for the ras p21.

In summary, our data show that ras mutations are uncommon in childhood thyroid cancers, and are only found in patients with extrathyroidal disease. The potential significance of this finding is limited by the small number of tumors which contained ras mutations. Further study is needed to determine if ras mutations are involved in the extrathyroidal spread of PTC.

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