The relative responsibility of each gene involved in this amplicon in carcinogenesis thus remains unknown. It is of interest to note that, in a rhabdomyosarcoma cell line, the amplicon did not include the MDM2 gene [16]. Further studies are needed to determine if one or several of these genes are also co-amplified in human breast cancer.

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Evidence Against Involvement of APC Mutation in Papillary Thyroid Carcinoma

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Papillary thyroid carcinoma (PTC) is one of several tumours associated with familial adenomatous polyposis (FAP), an inherited tumour syndrome which appears to result from germ-line mutation of the APC tumour suppressor gene. Here we investigate the possibility that somatic mutation of APC might play a role in sporadic PTC. 16 cases of PTC together with matched normal tissue were examined by single-strand conformation polymorphism (SSCP) analysis, concentrating on the mutation cluster region (MCR) of the APC gene (codons 1286–1513). No evidence of mutation was observed in any sample. We conclude that APC mutation, at least in the MCR, is not a significant causal mechanism in sporadic PTC.
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INTRODUCTION

PAPILLARY THYROID carcinoma (PTC) is the most common form of malignancy in the human thyroid. It is a differentiated neoplasm of follicular epithelium, with a distinctive morphology that occurs most frequently in women in the fourth and fifth decades of life. The aetiology of PTC is unknown, though the tumour is more common in populations with high dietary iodide intake [1] and in those who have been exposed to ionising radiation [2].

Activation of four oncogenes has so far been firmly implicated in the genesis of sporadic papillary carcinoma [3]; *ras* or *gsp*, activated by point mutation, and *ret* or *trk*, activated by chromosomal rearrangement. It has recently been shown [4], however, that these are nearly always found in isolation and are, therefore, presumably alternative rather than co-operating events. Since it is highly unlikely that any single activated oncogene can generate a clinical cancer, unknown co-operating events must be involved, which by analogy with other tumour types are likely to include loss of tumour suppressor genes. Unfortunately, cytogenetics and allelotyping have been disappointingly non-informative for PTC, the only reproducible abnormality being an inversion of chromosome 10 [5], which is now known to involve the *ret*

oncogene at that locus. Interestingly, mutation of the p53 tumour suppressor gene is extremely rare in PTC [6, 7].

Clues to the identity of tumour suppressor genes in a sporadic tumour type have often been derived from studying the corresponding inherited tumour syndrome, the same mutations often being found in both situations. For example, mutations of the APC gene for familial adenomatous polyposis coli (FAP) on chromosome 5q21 are also found in at least 60% of sporadic colorectal cancer [8].

Several reports have pointed to a familial clustering of PTC [9, 10], which may exist in as many as 6% of cases. As far as we are aware however, there has been no molecular genetic investigation of such families. PTC has also been recorded in patients with a number of other inherited tumour syndromes, such as ataxia-telangiectasia [11] and Peutz-Jehgers syndrome [12]. By far the best recognised association is with FAP, and indeed a common genetic basis for familial PTC and this syndrome was postulated over 20 years ago [13, 14].

Some 50 reports of patients with FAP and thyroid carcinoma have appeared in the literature [13–18], and while histological descriptions are often limited, the tumour type is usually stated to be papillary carcinoma.

Although the absolute risk of developing thyroid cancer in FAP is only approximately 2% [18], the relative risk is significant (though difficult to quantify). Two studies from polyposis registries have produced markedly different estimates, almost certainly because of different methods of analysis. Plail *et al.* [16] described four PTCs, all in young females, among 316 assessable patients with FAP in the records of the St. Mark's Hospital Polyposis Registry and on this basis, estimated the relative risk of developing PTC in FAP females under the age of 35 to be approximately 160. A much lower relative risk of 7.6 for thyroid cancer in all FAP patients, irrespective of age or sex, was reported from the Johns Hopkins Polyposis Registry [18]. Nevertheless, the findings that thyroid tumours in FAP tend to occur at a younger age [14, 16, 18] and are more frequently multi-focal [12–16] than in sporadic cases provides further support for a role of the APC gene in their pathogenesis.

Motivated by this evidence, we have now investigated the presence of APC mutations in sporadic PTC.

PATIENTS AND METHODS

Cases

16 cases of histologically-confirmed PTC were examined (Table 1), collected from laboratories in Cardiff, Bergen and Marseille (under the auspices of an EC Concerted Action into the molecular genetics of thyroid cancer). Two cases of follicular carcinoma were also included for comparison. In each case, histologically-normal thyroid tissue was also available as a source of germ-line DNA. Tissue was frozen rapidly in liquid nitrogen and held at −70°C prior to analysis.

Two colon tumour samples with known somatic mutations were also included as positive controls. Control 1 has a four base pair (bp) deletion at nucleotide 3924; control 2 has a C → T transition at 3982.

Table 1. Thyroid cancers studied for APC mutation

Case no.	Age	Sex	Diagnosis	Source
1	77	F	PTC	Bergen
2	29	F	PTC	Bergen
3	18	F	PTC	Bergen
4	35	F	PTC	Bergen
5	73	F	PTC	Bergen
6	51	F	PTC	Bergen
7	64	F	PTC	Marseille
8	46	F	PTC	Marseille
9	32	F	PTC	Marseille
10	62	F	PTC	Marseille
11	36	F	PTC	Marseille
12	33	F	PTC	Marseille
13	13	M	PTC	Marseille
14	40	F	PTC	Marseille
15	28	M	PTC	Cardiff
16	36	F	PTC	Cardiff
17	72	F	FTC	Marseille
18	43	F	FTC	Marseille

PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; F, female; M, male.

DNA extraction

DNA was extracted from pulverised frozen tissue by the guanidinium lysis procedure [19] or by the more recent method of Miller *et al.* [20].

PCR amplification

We confined our analysis to the so-called mutation cluster region (MCR) of the APC gene, between codons 1286 and 1513, which has been reported to harbour more than 60% of all mutations in colorectal tumours [21]. Primers were designed which covered 897 base pairs in four overlapping fragments from bases 3717 to 4614 (Table 2). PCR was carried out in 50-μl reaction volumes containing 100 ng of genomic DNA, 25 pmoles of each primer, 20 nmoles of each dNTP (Pharmacia Biosystems Ltd, St. Albans), 1.25 U of thermostable DNA polymerase and buffer consisting of 10 mMTris–HCl (pH 8.8), 50 mM potassium chloride, 1.5 mM magnesium chloride and 0.1% non-ionic detergent.

For sections 2 and 3, cycling conditions consisted of an initial

Table 2. PCR primers used to amplify the APC MCR in four overlapping segments

MCR section	Base pairs	Primers (5′ → 3′)
1	3717–4164	AAGTGGTCAGCCTCAAAAGG
		GTACATCTGCTAAACATGAGTGGG
2	3870–4301	TCAGACGACACAGGAAGCAG
		CTTGGTGGCATGGTTTGTC
3	4132–4423	CAGGAGACCCCACTCATGTT
		CAGCATTTACTGCAGCTTGC
4	4229–4614	GCAGTGGAATGGTAAGTGGC
		CATTTTCCTGAACTGGAGGC

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denaturation step of 5 min at 94°C, following by 33 cycles of 30 s denaturation at 94°C, 30 s annealing at 55°C (section 2) or 54°C (section 3), and 60 s extension at 72°C, with a final extension step of 9 min at 72°C. Section 4 amplification was similar, except that a 'hot start' was included (DNA polymerase added at 80°C, after the initial denaturation step) and annealing temperature was 53°C. For section 1, DNA polymerase was added at 80°C after a 5-min 95°C 'hot start'. One cycle of 30 s at 94°C, 30 s at 63°C and 1 min at 72°C was followed by similar single cycles with annealing temperatures of 60°C then 57°C, then 33 cycles with an annealing temperature of 55°C ending with a final 9-min extension at 72°C.

Single-strand conformation polymorphism (SSCP) mutation analysis

Five microlitres of PCR product were denatured for 5 min at 50°C in 1 μl 0.5 M NaOH, 10 mM EDTA. Three microlitres of stop solution (95% formamide, 10 mM EDTA pH 8, 0.01% bromophenol blue, 0.01% xylene cyanol) were added and the samples, including normal controls, loaded onto a 0.75 mm thick, 16 × 20 cm MDE gel containing 5% glycerol (SE600 system, Hoefer Scientific Instruments, Newcastle-under-Lyme, U.K.) and run at 20°C at up to 20 W. Gels were then silver-stained according to the manufacturers' instructions (Bio-Rad Laboratories Ltd, Hemel Hempstead, U.K.).

RESULTS

As expected, the two known mutant controls both showed abnormally migrating SSCP bands present in the tumour but not the normal samples, one of which is illustrated in Figure 1.

In contrast, no evidence of abnormal bands was seen in any of the 16 PTC samples analysed (either tumour or normal), nor in the two follicular carcinomas. Representative examples are shown in Figure 1.

DISCUSSION

In sporadic colorectal cancer, in which APC mutation is known to play a pivotal role, the majority (over 60%) of mutations occur in the so-called MCR which we examined here [21]. In this tumour type, up to 50% of cases have been shown to have MCR mutations as detected by either RNase protection or SSCP analysis [21] (the true rate is likely to be significantly higher since both techniques suffer from false negatives).

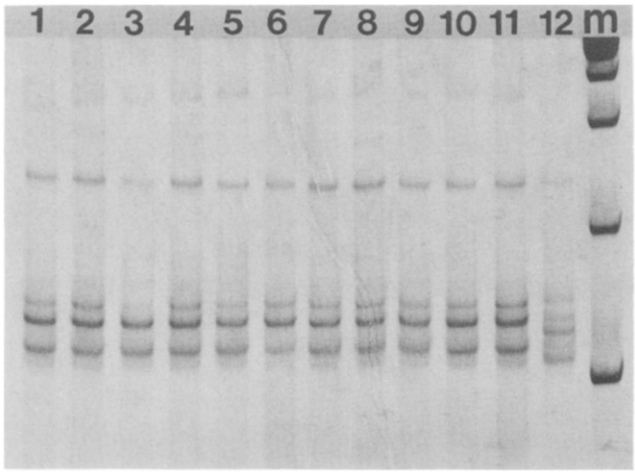

Figure 1. SSCP analysis of the APC MCR region in papillary thyroid cancer. Representative gel showing results for five thyroid papillary cancers and one colorectal positive control. For each case, tumour DNA (even-numbered lanes) is shown to the right of the corresponding germline DNA from matched normal thyroid tissue. Lanes 1–10: PTC cases (nos 1, 2, 7, 8 and 9); lanes 11 and 12: colorectal control (Con 1); lane m: size markers (1-kb ladder).

In contrast, no mutations in the MCR were found in the present study in 16 cases of sporadic papillary thyroid carcinoma. The probability of obtaining such a negative result if the prevalence of detectable mutations were similar to that in colon (e.g. 50%) can be estimated as $0.5^{16} = 0.000015$.

It is important, however, to point out that our series is still not large enough to exclude *any* involvement of APC in PTC. What we can say is that, taking the conventional statistical confidence limit of $P < 0.05$, the prevalence of detectable MCR APC mutations in these tumours is not greater than 17% [since $(1–0.17)^{16} = 0.05$], and is of course likely to be much lower. In other words, there is a 95% probability that the prevalence is less than 17% and a 90% probability that it is less than 13%.

We have, of course, only examined a small part (<10%) of the APC coding sequence, but there is no reason from studies of other tumour types, such as pancreas [22], to suspect that the distribution of mutations in thyroid could be sufficiently different to account for our negative findings. The most likely conclusion at present, therefore, is that, contrary to our original hypothesis, APC mutation is not a significant step in the development of sporadic PTC.

How can this result be reconciled with the apparent predisposition to thyroid cancer observed in FAP patients, the vast majority of whom would be expected to carry a germ-line APC mutation? The explanation probably rests on the different selective pressures operating for germ-line compared to somatic mutations in cancer.

For somatic events, provided there is no tissue-specific difference in the likelihood of a mutation arising (a reasonable assumption for APC given the diversity of effective mutation sites), the probability that a gene will be found to be mutated in a given tumour type depends on the strength of the selective growth advantage which it confers on that cell type, in comparison to that of other potential oncogenic events. In thyroid, in contrast to colon, we can conclude that APC mutation has a much weaker tumorigenic action than lesions such as *ret* and *trk* activation.

The situation is quite different, however, when one of the events is present in the germ-line. Even though too 'weak' to ever be selected as a somatic event, if already present it may be sufficient to greatly increase the effectiveness of subsequent somatic events, leading thereby to an increase in the probability of such events giving rise to a clinically detectable tumour. Furthermore it may modify the effect of, or select for a different set of, co-operating somatic events, hence generating a unique tumour phenotype. This possibility is strongly supported by the recognition that thyroid cancers associated with FAP, although often containing foci with 'classic' papillary architecture and nuclear morphology, nevertheless present other features more typical of follicular carcinoma—notably good demarcation and the presence of cribriform/solid and spindle morphologies (Dr R. Harach, in preparation). Indeed, it may be worthwhile repeating the present study with a meaningfully large series of follicular cancers.

We conclude that while APC mutation probably contributes to the development of the distinctive form of thyroid cancer seen in FAP, it is unlikely to be important in the common sporadic form of PTC.

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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