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Enhanced expression of EGF receptor and low frequency of *ras* mutations in X-ray-induced rat thyroid tumours

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Abstract Radiation is recognized as a carcinogenic factor for the thyroid gland. In this experimental study, oncogene expression was investigated in radiation-induced rat thyroid tumours. Forty 3-month-old Wistar rats received X-ray-irradiation to the neck region; 40 animals were untreated controls. After 14 months, thyroid tumours had developed in 25 of the 29 irradiated animals still alive; 76% of these tumours were considered malignant. No tumours developed in controls. Mutations of codons 12–13 and 59–63 of H-, K- and N-*ras* were analysed by PCR-SSCP (single-strand conformation polymorphism analysis) and sequencing of DNA from thyroid tissue. SSCP indicated a *ras* mutation frequency of 8%, but only one K-*ras* codon 12 (Gly-Cys) mutation was confirmed by sequencing. Protooncogene expression was analysed by mRNA slot blot hybridization analysis and immunohistochemistry. K-*ras* mRNA expression and EGF receptor mRNA and protein expression were significantly increased in the irradiated animals compared with controls, and in tumours versus nontumour tissue. This study of radiation-induced rat thyroid tumours demonstrates that *ras* expression may be subject to changes apart from activating mutations. Increased expression of EGF receptor in the tumours parallels the situation in human thyroid cancer.

Abstracts EGF receptor · *ras* · Radiation · Rat thyroid tumour

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Introduction

The causes of thyroid cancer are largely unknown, but radiation is recognized as a carcinogenic factor [35]. In humans, previous irradiation to the head and neck area has been associated with thyroid tumours, and especially carcinomas of the papillary subtype [16, 35, 37, 38]. The recent increase in thyroid cancer cases, especially among children, after the Chernobyl accident has focused the attention on radiation-induced thyroid neoplasia anew [31, 43, 48].

The molecular basis of thyroid cancer is currently emerging [47]. *ras* oncogene activation in various thyroid tumours has been reported in several studies [11, 17, 22, 23, 28, 42], and has been related to radiation exposure [4, 21, 49]. Thus, mutations of K-*ras* were detected in radiation-induced rat thyroid tumours [21], and an association between radiation and K-*ras* mutations was also suggested on the basis of earlier observations on a limited human material [49]. In a more recent report on human cases, *ras* mutations were detected in about 30% of radiation-induced thyroid tumours, but a similar frequency of mutation of all the three *ras* genes was observed [4]. On the other hand, a low frequency of *ras* mutations was found in both benign and malignant thyroid tumours from children exposed to radiation after the Chernobyl accident [31, 43]. This may be due to the special characteristics of these childhood neoplasms or, probably more importantly, to the papillary histological pattern, as *ras* mutations have been linked to follicular neoplasms [25].

While previous studies have focused on *ras* mutations, we also wanted to include data on *ras* expression in this study of X-ray-induced rat thyroid tumours. Furthermore, abnormalities of the epidermal growth factor receptor (EGFR) system have been observed in human thyroid cancer [15, 24], and we therefore examined the expression of EGFR, its ligand TGF-α and the related receptor neu in these experimentally induced tumours. Thus, the two key target genes, *ras* and *EGFR*, have been studied in the same thyroid tumours.

Table 1 Main histopathological diagnosis of rat thyroid tissue (*experimental group I* untreated controls, *experimental group II* animals treated with irradiation and MTU)

Experimental group	Number of animal	Surviving animals	*Histological group*			
			A (Normal thyroid follicles)	B (Diffuse hyperplasia)	C (Nodular lesions)	D (Carcinomas)
I	40	34	34			
II	40	29		4	6	19

Table 2 Primers used for PCR amplification of *ras* genes (*L* left primer, *R* right primer)

Mutation cluster region	L/R	Primer sequence	Fragment length	References
H-*ras* codon 12/13	L	5' AGT GTG ATT CTC ATT GGC AGG 3'		
	R	5' AAT GG TTCT GGA TCA GCT GG 3'	142 bp	–
H-*ras* codon 61	L	5' GAC TCC TAC CGG AAA CAG GT 3'		
	R	5' CTG TAC TGA TGG ATG TCT TC 3'	179 bp	[46]
K-*ras* codon 12/13	L	5' CCT GCT GAA AAT GAC TGA 3'		
	R	5' ATA GCA TCC TAG TAT AAG TAG GTG T 3'	118 bp	[11]
K-*ras* codon 61	L	5' GTA ATT CAT GGA GAA ACC TG 3'		
	R	5' TAT GTG TTT CTT TCG GGA GG 3'	112 bp	[10, 11]
N-*ras* codon 12/13	L	5' ATG ACT GAG TAC AAA CTG GT 3'		
	R	5' GCC TCA CCT CTA TGG TGG GT 3'	118 bp	[3]
N-*ras* codon 61	L	5' GTG ATT GAT GGT GAG ACC TG 3'		
	R	5' GGC AAA TAC ACA GAG GAA CCC TTC 3'	117 bp	[12]

Materials and methods

Animals and experimental design

A total of 80, 3-month-old Wistar rats (Munksgaard, Copenhagen, Denmark), 40 females and 40 males, were initially included. The animals were kept in plastic cages (2 animals/cage) and fed a standard pellet diet. Drinking water was given ad libitum. The rats were divided into two experimental groups: untreated controls (group I, *n*=40) and irradiated animals (group II, *n*=40). The NIH „Principles of laboratory animal care" were followed.

The experimental treatment model described by Christov [5] was used. From day 0, 0.1% methylthiouracil (MTU) was given in the drinking water to group II. On day 8, the animals in group II were anaesthetized with pentobarbital (1 µg/g body wt., i.p. injection) and irradiated in the neck region with a single dose of 300 cGy X-rays. The animals were protected by a whole-body lead shield with a circular opening (diameter 1.5 cm) over the thyroid gland.

The surviving animals (group I, *n*=34; group II, *n*=29) were sacrificed 14 months after irradiation. The left thyroid lobe was immediately frozen in liquid N_2 and stored at –80°C. This lobe was used for DNA and RNA extraction and analyses. The right lobe was removed together with the surrounding structures, fixed in 4% buffered formaldehyde and embedded in paraffin for standard histological examination and immunohistochemical analyses.

Seventeen animals died in the course of the experiment. Except in cases of early death related to anaesthetics and blood sampling, dead rats were subjected to autopsy. Tissue from such cases was processed for histological classification, but was not suitable for nucleic acid analysis or immunohistochemistry. A histological diagnosis was finally obtained for each of 69 cases (63 survivors and 6 autopsies, 3 from each experimental group). Immunohistochemistry and DNA/RNA analyses were performed on material from the 63 surviving rats.

The categories used for histological classification were modified after Napalkov [30], and the animals were grouped according to their histopathological diagnosis as follows: A, normal thyroid follicles (*n*=37); B, diffuse follicular hyperplasia: small follicles with hyperplastic epithelium (*n*=4); C, nodular (adenomatous) lesions (*n*=6), covering a spectrum from encapsulated solitary nodules (predominantly follicular adenomas) to multinodular (adenomatous) lesions, including nodules of possible hyperplastic nature; D, carcinomas (*n*=22): invasive growth in the tumour capsule, in the thyroid capsule, in striated muscle, and/or in vascular channels was used as criterion for malignancy. The histology data on the surviving animals are presented in Table 1. No tumours were found in control animals.

ras mutation analysis

The guanidinium lysis procedure was used for DNA and RNA extraction, as described by Aasland et al. [1]. Owing to the small size of the thyroid glands of untreated rats (group I), thyroid lobes from three or four control animals were minced and purified together. All the other samples were treated separately, giving a total of 37 RNA and DNA samples. DNA and RNA concentrations were determined spectrophotometrically.

DNA amplification was performed by the polymerase chain reaction (PCR), and the analyses were confined to the mutation cluster regions codons 12/13 and 61 (59–63) in all three *ras* genes. Primers were chosen to cover the areas of interest (Table 2) [3, 10, 11, 12, 46]. PCR was carried out in 100 µl reaction volumes containing 0.2 µg genomic DNA, 20 pmol of each primer and 20 nmol of each dNTP (Pharmacia Biosystems Ltd., St. Albans, UK), 10 µg BSA, 2 U Taq DNA polymerase (Gibco, UK) and buffer consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$. Cycling conditions consisted of an initial denaturation step of 5 min at 95°C, following by 35–40 cycles of 30 s at 95°C, 60 s annealing (temperature: see below) and 60 s at 72°C, with a final extension step of 10 min at 72°C. The following annealing temperatures were used: N-*ras* codon 12/13, 45°C; H-*ras* codon 61, 48°C; H-*ras* codon 12/13, 58°C; K-*ras* codon 12/13, K-*ras* codon 61 and N-*ras* codon 61, 55°C.

In addition to the rat DNA samples, PCR amplification was performed on DNA from plasmids, from murine cell lines and

Table 3 mRNA expression (mean±SEM) of *ras* genes, *EGFR* and *neu* in thyroid tissue from rats (n=63). mRNA expression was analysed by slot blot hybridization. The unit of expression is arbitrary (*Group I* untreated control animals, *Group II* animals treated with irradiation and MTU, *NS* not significant)

Gene	Group I (n=34)	Group II (n=29)	P-value
H-*ras*	0.91 (0.03)	0.66 (0.04)	0.0013
K-*ras*	0.61 (0.03)	1.48 (0.13)	<0.00005
N-*ras*	0.22 (0.004)	0.23 (0.01)	<0.00005
EGFR	1.06 (0.12)	1.42 (0.11)	0.018
neu	0.83 (0.07)	0.92 (0.05)	NS

from human cancer tissue known to harbour wild-type or mutated *ras* genes. Some of these controls were kindly provided by A. Ulvik, Haukeland Hospital, Bergen. A negative control consisting of PCR mixture with all ingredients except DNA was included in each run. All PCR products were checked on agarose gels.

The PhastSystem (Pharmacia Biotech) was used for mutation screening by single-strand conformation polymorphism (SSCP) analysis: 20% polyacrylamide gels (PhastGel homogeneous 20) were prerun for 100 Vh at 400 V and 25°C prior to loading. Equal amounts of PCR product and formamide were incubated for 2 min at 90°C and placed on ice, and 1 µl of this mixture was loaded onto the gel. Separations were run at 400 V for 100 Vh at 25°C for all PCR products except N-*ras* codon 61, for which they were run for 128 Vh at 20°C. Native buffer strips (Pharmacia) were used in all separations. Silver staining of gels was performed according to the PhastSystem manual. Wild-type and mutated controls were used for calibrating and optimizing the system, and were included in the runs.

DNA sequencing was performed by PCR amplification using biotinylated left or right primers, otherwise as described, on genomic DNA from all samples showing an abnormal migration pattern by SSCP analysis, and also on a number of normal controls. The two DNA strands were separated using alkaline denaturation and streptavidin-coated magnetic beads (Dynabeads from Dynal, Oslo, Norway), essentially as described in the manufacturer's protocol for template preparation. Sequencing was done by the chain-termination method using the Sequenase version 2.0 kit from USB (United States Biochemicals, Cleveland, Ohio). Reaction products were run on 6% urea gels and detected by autoradiography.

RNA slot blot hybridization

RNA was slot blotted onto nylon membranes as previously described [14]. Three slots of RNA were applied for each sample, containing 12, 6 and 2 µg RNA, respectively. For internal control purposes, some membranes contained samples from all series. Prehybridization and hybridization were carried out in 50% formamide at 42°C as described elsewhere [36]. DNA fragments were prepared from plasmids and ^{32}P-labelled ([α-^{32}P] dCTP from Amersham, Aylesbury, UK) using the oligo-labelling technique [9]. The following probes were used: H-*ras*, a 460-bp *Eco*RI fragment of BS-9 [7]; K-*ras*, a 382-bp *Sst*II/*Xba*I fragment of KBE-2 [8]; N-*ras*, a 3.4-kbp *Eco*RI fragment of pNRSac [27]; *neu*, a 1.6-kbp *Eco*RI fragment of pCER 204 [52]; c-*erb*B/EGFR, a 1.0-kbp *Bam*HI fragment of pE7 [51]; TGF-α, a 1.4-kbp *Eco*RI fragment of sp65C17N3 (Dr. R. Derynck, personal communication), and 28S rRNA, a 1.4-kbp *Bam*HI fragment of pA (I.L. Gonzales, personal communication). High sequence similarity between the corresponding human and rat genes was the reason for using fragments of cloned human genes as probes [2, 20, 40].

Analysis of autoradiograms was performed by densitometric scanning using an Enhanced Laser Densitometer (LKB products, Bromma, Sweden). The relative levels of expression of the genes specified above were estimated from scanning results as the amount of radioactive probe hybridized to each RNA sample relative to the amount of 28 S rRNA in each sample.

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections. The sections were heated for 1 h at 60°C and hydrated by transferring the slides through xylene and alcohol baths at room temperature. After 10 min in PBS, the sections were incubated with 0.1% proteinase K in PBS for 10 min at 37°C and then quickly transferred to PBS again for 5 min. Endogenous peroxidase was inactivated by incubating the sections in methanol with 1% H_2O_2 for 30 min at room temperature. For EGFR detection, antigen retrieval was performed by 10 min microwave cooking at this step, and proteinase K treatment was omitted. Incubations with normal serum and antibody preparations were performed as already described [14]. The following primary antibodies were used: the mouse monoclonal pan-ras antibody Ab-3 (cat. no. OP40, Oncogene Science, Manhasset, N.Y.), the rabbit polyclonal neu antibody sc-284 (Santa Cruz Biotechnology, Santa Cruz, Calif.), and the sheep polyclonal EGFR antibody 06-129 (Upstate Biotechnology, Lake Placid, N.Y.). All primary antibodies were used at a dilution of 1:50. Rabbit anti-mouse IgG (Dako, Copenhagen, Denmark) diluted 1:150 was used as secondary antibody for Ab-3, swine anti-rabbit IgG (Dako) diluted 1:100 for sc-284, and rabbit anti-sheep IgG (Vector, Burlingame, Calif.) diluted 1:100 for 06-129. All secondary antibodies were biotinylated. Colour development was by the avidin–biotin peroxidase method with DAB as substrate. The controls included replacement of the primary antibody with immunoglobulin of the same isotype, subclass and concentration, or with PBS. These controls were negative. Cognate peptide (sc-284P, Santa Cruz) was available for the neu antibody, and preincubation of the antibody with this peptide before use also completely abolished the staining. Paraffin sections of rat epidermal tissue (ras, EGFR) and rat kidney and lung tissue (neu) were used as positive controls.

Immunohistochemical positivity was subjectively graded as +++ (strong), ++ (moderate) or + (weak), according to the average staining intensity. Membrane staining and cytoplasmic reactivity were recorded separately.

Statistical analysis

Differences in mRNA levels between the two experimental groups were compared by the Student's *t*-test (Table 3).

Results

ras mutation analysis

All 37 rat thyroid DNAs were subjected to PCR amplification of codons 12/13 and 59–63 of all three *ras* genes, and the resulting 222 PCR products were screened for mutations by SSCP analysis (Fig. 1A). Eighteen PCR products showed an aberrant migration pattern, indicat-

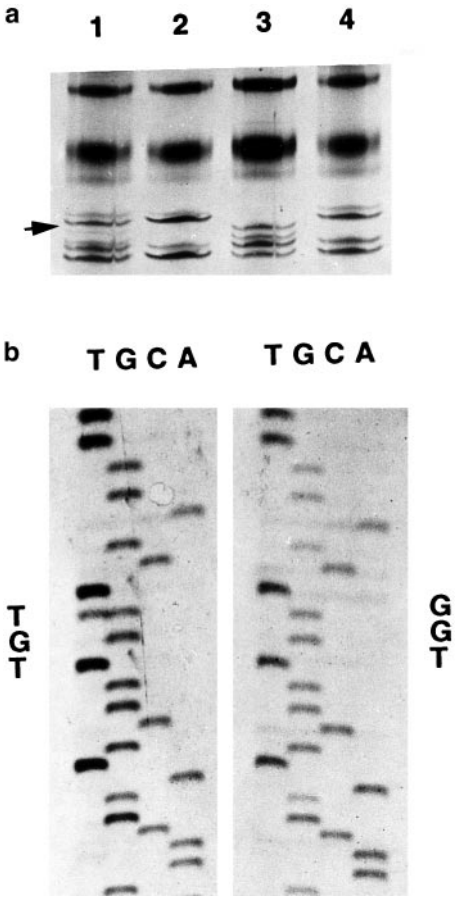

Fig. 1. a Mutation screening of K-*ras* exon 1 by DNA SSCP analysis. *Lane 1* rat thyroid tumour sample; *lane 2* normal rat thyroid tissue sample; *lane 3* mutated control; *lane 4* wild type control. *Arrowhead* points to upper extra band. **b** DNA sequencing of the rat samples in *lane 1* (*left panel*) and *lane 2* (*right panel*) is shown. Normal rat thyroid tissue contains normal K-*ras* codon 12 sequence GGT (*right panel*), tumour sample shows a TGT mutation (*left panel*). Tumour DNA also shows sequence of normal allele

ing mutation, and 6 additional samples were uncertain. These 24 samples were all amplified tumour DNA from irradiated animals and included the following amplifications: K-*ras* codon 61, 11 samples; K-*ras* codon 12/13, 2 samples; H-*ras* codon 61, 5 samples; N-*ras* codon 61, 3 samples; and N-*ras* codon 12/13, 3 samples. No mutations were suspected in the case of H-*ras* codon 12/13 amplifications. All samples showing an aberrant or uncertain migration pattern were sequenced, together with DNA from corresponding control samples. When DNA sequencing was performed, only one mutation was detected, a mutation of K-*ras* codon 12 (GGT→TGT, Gly→Cys), and only in one case (Fig. 1B). No mutations were found in codons 59–63 in any of the three *ras* genes, or in codon 12/13 of N-*ras*.

The mutated case did not differ from the other cases in histological features.

mRNA expression

The mRNA expression results are summarized in Table 3.

All three *ras* genes were expressed in all tissue samples analysed, and each gene exhibited a specific expression pattern in the two experimental groups. H-*ras* expression was significantly lower in irradiated cases than in untreated controls. N-*ras* mRNA expression, however, was the same in irradiated thyroids and untreated controls, whereas K-*ras* expression was significantly higher in the treated animals (group II). Complementary analysis comparing histological groups showed that K-*ras* mRNA expression was significantly higher in the tumour group (comprising histological groups C and D) than in the nontumour group (comprising histological groups A and B; P=0.0039). There was also a significantly increased K-*ras* expression in malignant versus benign tumours (P=0.0057).

Higher levels of *EGFR* mRNA were found in irradiated cases than in the control group, with increased expression in tumours compared with nontumour cases (P=0.0065). There was no significant difference between benign and malignant tumours. *neu* was expressed in all samples, but no significant differences were observed between the groups. TGF-α mRNA was not detected in any samples on hybridization of the slot blot membranes with a TGF-α specific probe.

Immunohistochemistry

ras protein expression in the thyroid is investigated using a pan-ras antibody. Both the degree and the extent of the immunostaining vary within both experimental groups. Single negative cases were observed in both groups, but the majority of samples showed some reactivity in the cytoplasm, often combined with distinct membrane staining. Membrane reactivity tended to be more prominent in the carcinomas, especially in areas with papillary differentiation, but no statistically significant difference was found between the experimental groups. As this initial screening only revealed low or moderate pan-ras protein expression levels in the samples, additional investigations using antibodies recognizing the individual ras protein isoforms were not performed.

EGFR immunoreactivity was detected in the majority of cases. Sections from control animals typically exhibited weak cytoplasmic staining but no membrane reaction, and some cases were completely negative. In group II, a highly significant increase of EGFR membrane staining was observed (P<0.00005) compared with the control group (Fig. 2). Membrane reactivity, generally of weak to moderate intensity, was increased in tumours compared with nontumour tissue (P<0.00005), and in malignant versus benign tumours (P=0.0004). Carcinomas with a papillary growth pattern showed the most prominent EGFR membrane staining, but cytoplasmic EGFR staining intensity was also generally increased in the tumour tissue (Fig. 2).

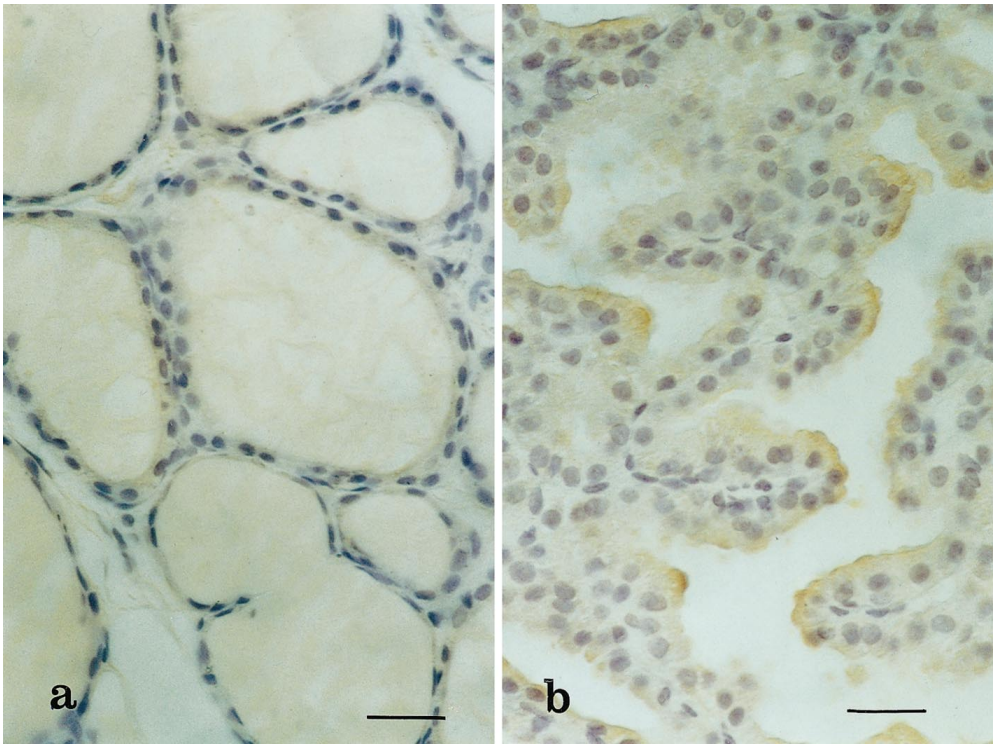

Fig. 2a, b Immunohistochemical demonstration of EGFR in rat thyroid tissue. **a** Normal thyroid tissue is negative or shows very weak cytoplasmic staining, with no membraneous reactivity. **b** Thyroid carcinoma. EGFR reactivity is present as patchy membrane staining and some cytoplasmic reactivity in the tumour epithelium. ABC peroxidase, ×560. *Bar* 20 μm

neu immunoreactivity was present in all samples analysed, and cytoplasmic staining and some membrane reactivity were observed. No significant differences in staining intensity or staining patterns were observed between the two experimental groups or between tumour and nontumour cases.

Discussion

The present work was performed to study radiation-induced tumorigenesis in the thyroid gland using an experimental rat model adapted from Christov [5]. Histopathological examination of the animals clearly demonstrated a functioning model, with general follicular hyperplasia of the thyroid tissue and tumours in most of the irradiated animals. Using the present criteria, 76% of the tumours were classified as carcinomas. The promoter effect of elevated TSH levels obtained by goitrogen (MTU) administration probably shortened the latency period for the appearance of tumours [50]. Several rats, especially those with locally advanced thyroid tumours, died from their disease before the end of the experiment.

Involvement of the *ras* genes is frequently seen in human tumours [18] and has been reported as an early and important event in thyroid tumorigenesis, in man as well as in animal models [22, 29]. The *ras* genes may be activated by point mutations or by gene amplification in the thyroid [28], and high frequencies of mutation of all three *ras* genes were reported in the first studies on *ras* involvement in human thyroid tumours [22, 23, 43]. However, later reports are not consistent on these matters concerning either the frequency or the nature of the mutations [11, 13, 17, 25, 28, 29], and no final consensus has yet been reached on the role of *ras* in thyroid tumorigenesis. Nevertheless, *ras* seems to be involved in the development of follicular and undifferentiated carcinomas, but rarely in papillary carcinomas [25].

In the present work, a striking lack of *ras* mutations was observed, as only one mutation was clearly demonstrated by DNA sequencing. This mutation was a K-*ras* codon 12 mutation (Gly→Cys) (Fig. 1), which was found in a carcinoma with papillary and solid areas. To our knowledge, this Gly→Cys mutation has not previously been described in rodent thyroid tumours, though it is observed in humans.

The radiation source and additional experimental conditions may explain some of the differences concerning the observed frequency and nature of *ras* mutations in different experimental models. In their study on thyroid tumours in rodents, Lemoine et al. [21] used i.p. radioiodine and no external radiation. Of additional importance in humans, population differences as well as variations in tumour classification criteria may influence mutation frequencies [25, 39]. Furthermore, the majority of studies on *ras* mutations in thyroid tumours have been performed using oligonucleotide probe hybridization [11, 13, 17, 22, 23, 28, 29, 39, 49]. This technique requires particularly stringent hybridization and washing conditions to avoid false-positive results [25]. In the present study, PCR-SSCP analysis was used for mutation screening [33, 45]. This method has also been applied to thyroid tumours in another study [34]. In the above reports [33, 34, 45] neither false-negative nor false-positive re-

sults were evident. In our hands, the method indicated 8–11% of mutated cases in irradiated animals. However, we were not able to verify this number by DNA sequence analysis. The increased SSCP sensitivity may be due to the use of 20% instead of 6% polyacrylamide gels. Alternatively, silent mutations in other codons of the amplified exon may have changed the migration pattern in the gel, giving false-positive cases. However, although we focused primarily on the mutation cluster regions when sequencing, the sequence of the whole fragment was recorded, and no other mutations were obvious.

SSCP mobility shifts were observed in all mutation-positive control DNA samples. We cannot, however, completely rule out false-negative cases either, as normal background caused by nontumour cells in the biopsies may obscure the interpretation and detection of mutations. This „background" probably interferes more with DNA sequencing than with SSCP. DNA sequence analysis is also a sensitive technique, but probably a minimum percentage of the cells must contain the mutation to make it distinguishable from the normal allele on a sequencing gel. On the other hand, if only a minor fraction of the tumour cells harbour the mutation, this may call in question its biological consequences. Taken together, only one *ras* mutation was verified by DNA sequencing, but the frequency of *ras* mutations in the irradiated animals is probably higher, as indicated by PCR-SSCP. However, we have no reason to believe that the true frequency of mutations exceeds 8%, which is lower than previously reported [21–23].

All three *ras* genes were expressed in all samples, according to analysis by RNA slot blot hybridization. Owing to varying degrees of full-length RNA in our preparations, Northern blot analysis was not suited for gene-specific mRNA quantitation. The slot blot technique was therefore used to obtain reliable data [36]. The different probes were all shown by Northern analysis to be highly gene specific without any detectable nonspecific hybridization. Despite a low mutation frequency, significantly increased K-*ras* expression was evident in irradiated animals, and in tumours compared with nontumour thyroids, with a major increase in the malignant cases. Similarly, the expression of H-*ras* was changed, although to a lesser extent (Table 3). These findings indicate that *ras* expression changes during tumour development and that the individual *ras* genes react differently. Increased *ras* expression without the presence of *ras* mutations has been observed in several human tumours, such as breast carcinomas [26], colorectal carcinomas [19], and head and neck squamous cell carcinomas [54], and it has been shown that p53 protein [56] and steroid hormones [55] may act as transcriptional regulators of H-*ras* expression. The insignificant variations in total ras protein probably reflect the net effect of increased and decreased individual expressions. However, the changes in cellular ras functions cannot be predicted from the overall protein levels, as the three different ras proteins may have distinctly different functions in signal transduction, i.e. the three ras forms may be optimally coupled to different receptor pathways [53].

EGF is recognized as a growth stimulator for thyroid cells in nearly all species tested [32], with the rat as a hitherto apparent exception. No EGF-binding could be detected in the immortalized rat follicular cell line FRTL-5 [6], and EGF stimulation elicited no growth response in this cell line or in primary rat thyroid cell cultures [41], leading to the common belief that rat thyroid tissue did not express EGFR. However, Sugawa et al. [44] have demonstrated (by immunoprecipitation) that FRTL-5 cells do possess EGFR, and that the tyrosine kinase of the receptor is activated during cell proliferation. In the present work, EGFR expression in rat thyroid tissue was verified by detection of both mRNA and protein. EGFR expression was demonstrated in normal controls, and increased expression was evident in tumour tissue (Table 2), most markedly in malignant tumours, with increased EGFR mRNA as well as increased protein levels. These findings are in line with observations on human thyroid tumours, which overexpress EGFR compared with nontumour thyroid tissue [15, 24].

In human papillary carcinomas, evidence for a TGF-α–EGFR autocrine loop has been demonstrated [15]. However, no TGF-α mRNA was detected in the present material. This finding does not exclude stimulation of EGFR by TGF-α in the rat thyroid, but an autocrine mechanism is improbable. Expression of *neu* was present in all samples, but no significant variations were observed between the two groups in this experimental model.

In conclusion, these specimens from radiation-induced rat thyroid tumours demonstrated small, yet significant variations in *ras* and *EGFR* expression. *EGFR* expression was up-regulated in tumour tissue compared with normal controls, which parallels observations on human thyroid material [15, 24]. A significant increase of K-*ras* mRNA expression was observed in the thyroids of irradiated animals, while there was a low frequency of *ras* mutations. These findings suggest that *ras* gene expression may be subject to functional changes apart from activating mutations.

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