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Differences in the Susceptibility to Iodine^{131}-induced Thyroid Tumours amongst Inbred Mouse Strains

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***M. m. Molossinus*/Radioiodine/Adenoma/Carcinoma/Classification.**

Genetic factors can modify susceptibility to the carcinogenic effect of ionising radiation. To establish if radioiodine-induced thyroid cancer is similarly genetically influenced, we studied F1 hybrid crosses between inbred mouse strains. Mice were perinatally exposed to iodine-131 and thyroid tissues examined after 18 months. Differences in the incidence and distribution of histological subtypes were quantified in relation to genetic background. As expected, the occurrence of thyroid lesions was significantly higher in irradiated mouse hybrids than in unirradiated controls. The most frequent alterations were the simple and the complex hyperplasias, followed by follicular adenoma and, less frequently, follicular carcinoma. Both the incidence and distribution of the histiotype were different between the hybrid mouse crosses. Crosses using JF1 mice (*M. m. molossinus*) produced F1 offspring that were more resistant to radiation-induced thyroid lesions. Sequence analysis of *Braf, Ret, Hras, Kras, Kit* and *Trp53*, all genes that are commonly mutated in human thyroid cancers, did not show any evidence of mutation in the tumours. However, microsatellite analysis of genomic DNA revealed frequent allelic imbalances in complex hyperplasia and follicular adenoma. We conclude that genetic background, in particular the JF1 genotype, confer differences in susceptibility to the carcinogenic effects of radioiodine on the thyroid.

INTRODUCTION

Increased incidences of thyroid cancer occur in populations exposed to radioiodine that has been released as a consequence of atomic bomb detonations or nuclear accidents such as the Chernobyl power plant explosion.[1,2)] In particular children and adolescents are at greater risk due to greater incorporation of radioiodine, as seen in the aftermath of the radioactive fallout after the Chernobyl accident.[3)] Epidemiological studies indicate a significant association of thyroid cancer with exposure to iodine-131 especially among individuals younger than 18 years.[4,5)] External radiation exposure is also a potent inducer of thyroid cancer, as shown in children treated with external beam radiation to the head.[6)]

There is evidence indicating that individual genetic background can influence disposition to non-medullary thyroid cancer (NMTC). Although the majority of thyroid carcinomas arising from follicular cells appear sporadic, a genetic component is indicated by the familial accumulation of thyroid cancer seen in about 5% of all NMTC cases.[7)] Familial non-medullary thyroid cancer (FNMTC) is a very heterogeneous disease that divides into two main groups.

One group comprises those familial cancer syndromes that are characterised by a range of non-thyroidal neoplasms in association with NMTC, including Gardner syndrome (FAP), Cowden disease, Carney complex and Werner syndrome (for a review see 7,8).

The other group of FNMTC is characterised by the predominance of thyroid carcinoma of follicular cells, although other neoplasms may occur.[7)] This single tumour entity is observed to be more aggressive than apparently sporadic forms of NMTC.[9)] The FNMTC syndrome displays features of clinical anticipation, with an earlier age at disease onset and increased severity in successive generations.[10)] Several attempts have been made to identify the genes responsible for FNMTC, using genome wide linkage analysis.[11,12)] Although candidate loci have been indicated, the causal gene has not been conclusively identified. To investigate the role of predisposing genetic factors in radiation-associated thyroid carcinogenesis we have adapted a mouse model that

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recapitulates the radioiodine exposure scenario of the Chernobyl accident.

Mouse thyroid gland proliferations are generally classified into hyperplasias, adenomas and carcinomas, with qualification of the growth pattern (follicular, papillary or solid) added when appropriate. However, no standard scheme exists to apply this classification system, nor to relate the histopathology to that seen in human thyroid cancers.[13–16] In contrast, the classification of human thyroid lesions follows clearly defined morphological characteristics (growth pattern, nuclear features and capsule) defined by the WHO. These features mainly define the different entities of thyroid neoplasms, the follicular adenomas (FTA) with normo-, macro-, microfollicular and solid subtypes; and the carcinomas, subclassified as follicular (FTC) and papillary (PTC) thyroid carcinomas. To relate our mouse studies to human situation we have adapted the human WHO classification system[17–19] to the histological analysis of the mouse thyroid sections.

In thyroid carcinogenesis a high rate of *BRAF* and *RAS* gene mutations are identified, as well as various *RET/PTC* gene rearrangements. *BRAF* mutations are a prominent DNA change in thyroid carcinomas and were found in up to more than 55% of PTC.[20] The predominantly diagnosed thyroid tumour type after the Chernobyl accident was PTC,[2] however, *BRAF* mutations are rare in post-Chernobyl cases and *RET/PTC* rearrangements were found at a high prevalence.[21] Recently FOXO3a shown to exhibit different localization in thyroid carcinoma and normal thyroid cells suggesting a novel pathomechanism involving cellular stress response.[22] In human post-Chernobyl PTCs a radiation-specific molecular marker of thyroid cancer was recently identified, a gain on chromosome 7 that was associated with prior radiation-exposure.[23] In this study of mouse thyroid tumour incidence we report that strain-dependent differences exist in the incidence and distribution of thyroid lesions after exposure to radioiodine. No characteristic genetic markers could be identified in the thyroid lesions.

MATERIALS AND METHODS

Animal breeding and maintenance

Mice were obtained from Helmholtz Zentrum München breeding stocks. Hybrid F1 mice were obtained from matings of C3H female × C57BL/6 male (C3B6F1), BALB/c female × C3H male (CC3F1) and JF1 female × C3H male (JFC3F1). In C3B6F1 and CC3F1 hybrids the litter size of radioiodine exposed breedings was about half of the litter size of control mice. JFC3F1 hybrids showed similar litter size in radioiodine exposed and unexposed control mice. All experiments and housing of the animals were performed under a licence granted according to the German Law on the Protection of Animals (Regierung von Oberbayern AZ 211-2531-53/01).

In vivo administration of iodine-131

The F1 hybrid mice were exposed to radioactive iodine during embryonic and neonatal growth.[24] Adult females were fed an iodine-deficient diet (C1042, Altromin, Lage, Germany) for 6 weeks before mating and were subsequently injected i.p. with iodine-131 (2 × 100 kBq per 20 g mouse) on day 16 post conception and again on day 7 post partum. The offspring incorporate iodine-131 from the mothers during foetal development and after birth with the milk. The mothers of control animals were also maintained on the iodine-deficient diet, but did not receive iodine-131 injections. Three weeks after birth the young F1 hybrid mice were weaned and were given access to normal mouse diet and water *ad libitum*. The incorporated radioactivity of the offspring was measured using a dose rate meter (β-γ detector, UMo LB123, EG&G Berthold Technologies, Bad Wildbad, Germany). Although there was some individual variation in incorporated activities, no systematic differences between the different F1 hybrid strains were observed. Mice were sacrificed if they showed signs of disease or at the end of the 18 months follow-up period.

Histology and immunohistochemistry

At necroscopy thyroid tissue was excised and fixed in neutral buffered formalin (4%) for 48 hours prior to embedding in paraffin. Transverse 2–4 μm sections were collected on glass slides and stained with haematoxylin and eosin or a standard Masson's trichrom staining was performed. Histological features were classified according to the WHO-Classification of Tumours[17–19] adapted to accommodate the characteristics of mouse morphology[25] (Table 1). Two clinical pathologists (LQM, JCW) and one veterinary pathologist (GH) evaluated each animal.

For immunohistochemical staining 1–2 μm sections were deparaffinised and heated for antigen retrieval in a microwave pressure cooker filled with citrate buffer for 30 min at

Table 1. Comparison of thyroid lesion classification in mouse and man.

thyroid lesion	Conventional mouse thyroid nomenclature	Nomenclature of the human WHO classification
Hyperplasia	cystic subtype	Simple hyperplasia: colloid and parenchymatous goitre
Adenoma	papillary, follicular and solid subtypes	FTA: normo-, micro-, macro-follicular, and solid and atypical adenoma
Carcinoma	papillary, follicular and solid subtypes	FTC: minimally and widely invasive PTC
	anaplastic carcinomas	anaplastic carcinomas

1000 Watt. The sections were blocked with 3% serum in TBS buffer and incubated overnight at 4°C in a humidity chamber with the primary antibody. An automated immunohistochemistry slide staining system (Ventana Medical System, CA, USA) was used for further proceedings according to the manufacturers recommendations.

Statistical analysis

When not otherwise mentioned, the Chi-square test (χ^2-test) or Fisher's exact test were used for statistical analysis. Significances at a p-value of less than or equal to 0.05 are reported.

Laser capture microdissection and DNA isolation

Thyroids sections were cut from paraffin blocks, collected on glass slides and covered by a polyethylene membrane (Membrane Slides, P.A.L.M. GmbH, Bernried, Germany). The sections were deparaffinised, stained with haematoxylin and the areas of interest were microdissected and recovered (MicroBeam Palm®, Microlaser Technologie, Bernried, Germany). With this technique the areas of thyroid tumour tissue were separated from the surrounding normal tissue of thyroid, muscle, trachea and oesophagus. DNA was extracted from microdissected thyroid sections using the QIAamp Mini Kit (Qiagen GmbH, Hilden, Germany).

DNA sequencing

Genomic DNA from microdissected thyroid tissue samples was amplified in a standard PCR reaction using the GoTaq Green Master Mix (Promega, Mannheim, Germany) with primers (Table 2) covering the mutational hotspots of *Braf, Ret, Hras, Kras, Kit* and *Trp53*. PCR products were cleaned with the exonuclease/alkaline phosphatase method (enzymes and protocol from Fermentas, St. Leon-Rot, Germany) and sequenced using the Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

Microsatellite analysis of allelic imbalance

Genomic DNA from 35 C3B6F1 and 7 CC3F1 hybrid animals were analysed for allelic imbalance. Two DNA samples were obtained from each mouse, one sample was from microdissected thyroid tissue (8 NAD, 14 SH, 12 CH, 6 FTA and 1FTC from iodine-131-exposed mice and one FTA form an unexposed mouse) and the other sample from tail tip (control non-tumour tissue). Microsatellite markers (27 informative markers for C3B6F1 and 8 informative markers for CC3F1) on different chromosomes were amplified in standard PCR reactions. The PCR products were separated in a 3% agarose gel by electrophoresis, stained with ethidium bromide and photographed. For analysis of allelic status the amplification products of thyroid tissue and tail tip DNA from the same mouse were compared. Allelic imbalance was defined as total loss or a strong reduction of the signal of one allele in the stained agarose gel.

RESULTS

Histological evaluation

The normal murine thyroid (Fig. 1A) shows considerable

Table 2. Primers used for PCR and sequencing.

primer[a]	forward	reverse
Braf exon 14	CTCTTTTGTTTGCCTTGGCT	GCCAATGGCATCGTGTTATAC
Braf exon 18	ACCTGAAATCTTCAAAATGCTT	AAGCCCTTCAGTGTATTTCTCG
Ret exon 10	GCTACTGACTCCGAGTTGGG	CCCTTGGTGGACCTCATAAA
Ret exon 11	GCACCCGGTCTGTAACATCT	GCCATGTTTCCTCCAACATT
Hras exon 2, 3	GGTCAGGCATCTATTAGCCG	CACACGGAACCTTCCTCAC
Kras exon 2	TTTTTATTGTAAGGCCTGCTGA	TGATATCTTTTTCAAAGCGGC
Kras exon 3	GCTTTGCCTGTTTTGAATGG	TGCAGGCATAACAATTAGCA
Kit exon 11	GCGGGTGACACATCTTTCTT	AAGACAAAGGAAGCCACTGC
Kit exon 17	GGAGAGCTGAAATGAATGGC	CTGGATACCAAAGCACCCTG
Trp53 exon 5	ACACCTGATCGTTACTCGGC	GAATAAGTCAGAAGCCGGGA
Trp53 exon 7	GTAGGGAGCGACTTCACCTG	GGGACTCGTGGAACAGAAAC
Trp53 exon 8	TGCTGGTCCTTTTCTTGTCC	GAGCAAGAGGTGACTTTGGG

[a] name of the primer stating the target gene and exon

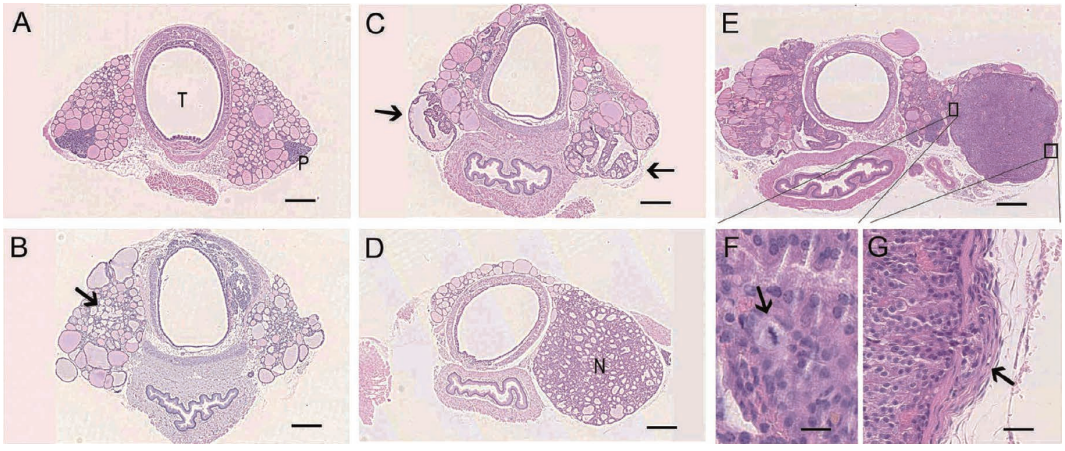

Fig. 1. Thyroid lesions from hybrid mice (A–D: C3H × C57BL/6, E: JF1 × C3H), histological sections, H&E stained. **A:** Normal thyroid gland (NAD - no abnormality detected), demonstrating typical follicles lined by simple cuboidal epithelium. Parathyroid gland (P). Lumen of trachea (T). **B:** Simple hyperplasia (SH), characterised by variation in follicular size with coalesce of contiguous follicles (arrow) lined by flattened epithelium. **C:** Complex hyperplasia (CH), note the papillary projections into the follicular lumen (arrows). The foci of hyperplasia are not encapsulated and there is minimal compression of the adjacent normal tissue **D:** Follicular thyroid adenoma (FTA), formed by proliferation of follicular cells in a well-circumscribed nodule (N). **E:** Follicular thyroid carcinoma (FTC), clear nodular formation with capsule and compression of the adjacent thyroid tissue. The left box (**F**) shows the area with elevated rate of mitosis and the right box (**G**) shows penetration of the capsule and blood vessels. Depicted with higher magnification in **F** and **G**. (**A–E**: H&E staining, 5× magnification, bar = 100 µm. **F–G**: H&E staining, 40× magnification, bar = 12.5 µm).

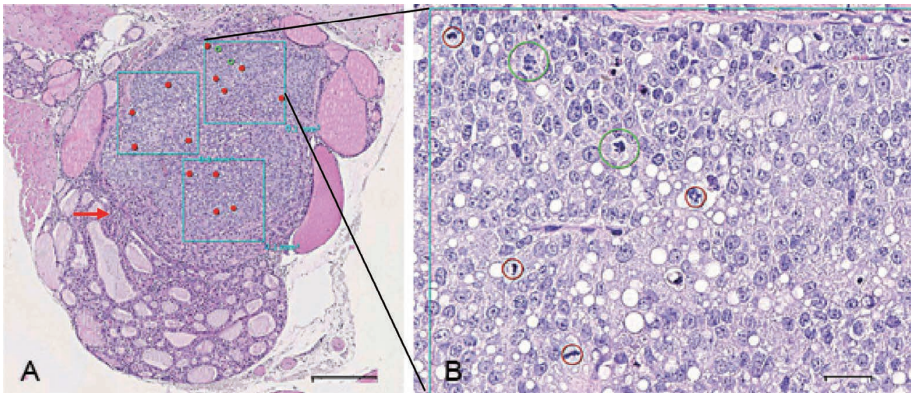

Fig. 2. Follicular thyroid carcinoma (FTC) diagnosed in a JF1 × C3H hybrid mouse. Note the nodular formation with capsule and compression of the surrounding tissue (red arrow). **A:** Quantification of numbers of mitosis in 3 different areas (with more than 4 mitotic figures in 0.1 mm²). B: Higher magnification of the upper right quadrat in A with 4 mitosis (red circles) and 2 atypical mitosis (green circles). (H&E staining, A: 5× magnification, bar = 250 µm. B: 40× magnification, bar = 25 µm).

variation in the size, shape and pattern of follicular epithelium that is depending on the strain and age of the mice. The normal thyroid morphology of CC3F1 (BALB/c × C3H) and JFC3F1 (JF1 × C3H) hybrid mice was dominated by large follicles, whereas the follicle sizes in C3B6F1 (C3H × C57BL/6) mice were more variable.

Simple hyperplasia (Fig. 1B) was characterised by diffusely distributed macrofollicular changes, without the presence of nodules. The hyperplastic thyroid epithelium was typically flattened (colloidal goitre) or hypertrophic (parenchymatous goitre), with basally located nuclei.

Complex hyperplasias (Fig. 1C) showed, in addition to the changes of SH, the formation of multiple nodules, which were circumscribed, but not encapsulated, and caused no compression of the surrounding thyroid tissue. The growth patterns of the nodules in complex hyperplasia included forms with solid clusters, papillary infoldings into the follicular lumen, or follicular nodules with variable follicular sizes.

Follicular thyroid adenomas (Fig. 1D) were characterised, in accordance with the human classification scheme, by single, circumscribed and encapsulated lesions, compressing the surrounding tissue; their abnormal architecture was dissimilar from the remaining healthy parenchyma. Murine fol-

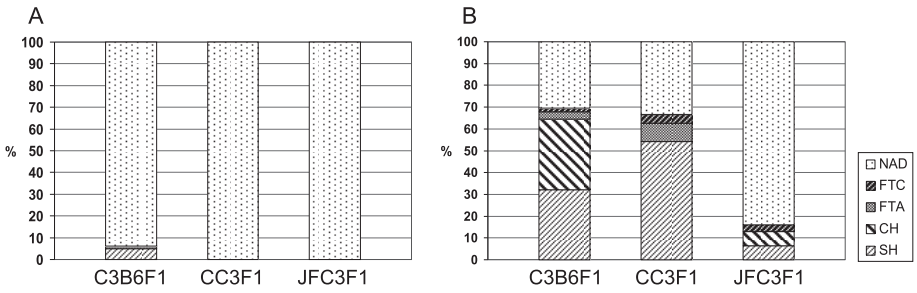

Fig. 3. Distribution of hyperplastic and neoplastic thyroid lesions in different hybrid mice from F1 crosses of C3H × C57BL/6 (C3B6F1), BALB/c × C3H (CC3F1) and JF1 × C3H (JFC3F1). **A**. Unirradiated control mice showed mostly normal thyroids (NAD - no abnormality detected) and developed thyroid lesions only at very low numbers. **B**. The iodine-131 treated mice showed significantly more simple hyperplasia (SH), complex hyperplasia (CH), follicular adenoma (FTA), follicular carcinoma (FTC) and less normal thyroids (NAD – no abnormality detected).

licular adenomas showed a weaker stromal response (fibrosis and inflammation) compared to human lesions, resulting in a thinner capsule. As capsule formation is an important feature that distinguishes between hyperplastic nodules and adenoma Masson's trichrome staining for fibrous tissue was used diagnostically.

Thyroid carcinomas are histologically subdivided into papillary and follicular carcinoma in human schemes. In our study scattered PTC-like cellular changes were observed in a number of carcinomas, especially in solid variants, but were only present in isolated cells. As these carcinomas lacked characteristic nuclear PTC features (e.g. ground glass nuclei, pseudoinclusions, and nuclear grooves) we were unable to classify them as PTC. All mouse carcinomas were therefore classified as FTC (Fig. 1E). Local invasive behaviour with penetration of the capsule and blood vessels was observed in most of the carcinomas studied, extensive infiltration of other tissues was rare and no evidence for distant metastasis was found. All murine FTCs showed a characteristically elevated mitosis rate with atypical mitotic figures (Fig. 2). With the exception of one case, all FTCs were present in tissue with a background of the proliferative changes associated with hyperplasias or FTA.

Influence of radioiodine exposure on the thyroid

The thyroid glands of 228 mice (114 iodine-131-exposed and 114 control mice) were analysed. Pathological alterations were found in the thyroid tissue of 54.4% (62/114) of the iodine-131-exposed mice but in 4.4% (5/114) of the control mice. Solitary thyroid cysts were present in 4.4% (5/114) of the iodine-131-exposed and in 3.5% (4/114) of the control thyroid glands. No other pathological findings were seen in the non-exposed thyroid glands (110/114), and there was no correlation of cyst development with radioiodine uptake in the exposed animals.

Compared to the group of unirradiated control mice, the iodine-131-exposed mice showed a significantly higher incidence of thyroid lesions, namely simple hyperplasia (SH), complex hyperplasia (CH), follicular thyroid adenoma (FTA) and follicular thyroid carcinoma (FTC) (Fig. 3). The increase of lesion frequency was statistically significant using the chi-square test for analysis (Table 3).

Table 3. Pathological lesions in the thyroid sections of iodine-131-exposed and control mice. Statistical significance was analysed with the chi-square test.

thyroid lesion[a]	control mice (n = 114)		I^{131}-exposed mice (n = 114)		chi square test (p-value)
	n	%	n	%	
NAD	109	95.6	52	45.6	0.00143
SH	4	3.5	34	29.8	1.12E-22
CH	0	0.0	21	18.4	5.64E-26
FTA	1	0.9	4	3.5	1.32E-06
FTC	0	0.0	3	2.6	5.13E-08

[a] abbreviations: NAD - no abnormality detected, SH - simple hyperplasia, CH - complex hyperplasia, FTA - follicular thyroid adenoma, FTC - follicular thyroid carcinoma

The simple hyperplasias were characterised by macrofollicular changes without formation of nodules, and an enlargement of the thyroid gland (goitre). In 38 mice we observed simple hyperplasia, 34 (29.8%) in the iodine-131-exposed group and 4 (3.5%) in the control group. Complex hyperplasia with formation of multiple variably-sized nodules was found in 21 of 114 (18.4%) iodine-131-exposed mice but was not present in any of the 114 unexposed control thyroids. Five follicular thyroid adenomas were diagnosed, of which four cases developed in I-131 irradiated mice and one in an unirradiated control mouse. Coexistence of FTA and FTC was seen in one mouse from the iodine-131-exposed group. Follicular thyroid carcinomas (FTC) were found in 3 (2.6%) of the iodine-131-exposed and in none (0%) of the control mice. A solid growth pattern was observed in all FTCs.

Thyroglobulin, calcitonin and Ki67 immunohistochemistry

Immunohistochemistry using thyreoglobulin and calcitonin antibodies was performed on all carcinomas with a solid growth pattern to verify the cellular origin of the tumour (follicular epithelial cells or c-cells) (Supplementary Figure). Proliferation activity was established by elevated Ki67 expression in the tumour cells of FTCs (Supplementary Figure). Two antibodies (cytokeratin 19, galectin-3) usually used to prove PTC in human thyroid sections did not show cross-species reactivity on the murine sections of FTA and FTC and could not be used.

Strain-dependent differences in the development of sporadic thyroid lesions in non-irradiated mice

Among the unirradiated control mice in our study only C3B6F1 mouse hybrids showed any evidence of spontaneous thyroid lesions (3 SH and 1 FTA). In contrast the CC3F1 and JFC3F1 control mice developed no thyroid lesions (Table 4). No significant strain differences in the incidence of thyroid lesions were found in the non-irradiated control mice.

Strain differences in the development of radiation induced thyroid lesions

In the radioiodine-exposed group, the proportion of mice affected by thyroid lesions varied considerably between the different hybrid strains. Normal thyroids were observed after irradiation in 31% C3B6F1 and 33% CC3F1 hybrid mice, whilst 84% of the irradiated JFC3F1 hybrids showed normal thyroids (Fig. 3). The significantly lower total incidence of thyroid lesions in the JFC3F1 mice ($p < 1E\text{-}3$) suggests a genetically-determined resistance to radiation-induced thyroid lesions.

Strain differences were also seen in the distribution of the various thyroid lesions of I-131-exposed mice. Interestingly, SH were observed at a significantly higher rate in CC3F1 mice compared to C3B6F1 and JFC3F1 mice ($p < 2E\text{-}3$) but no progression to CH was observed. Because of the overall small numbers of thyroid tumours it is not possible to infer a clear strain difference for FTA and FTC.

Sex distribution of thyroid lesions

The thyroid changes were analysed for sex-specific effects on distribution (Table 4). Hyperplastic lesions occur at a slightly higher rate in female mice in both I-131-exposed (females 28.1%, males 20.2%) and unexposed groups (females 2.6%, males 0.9%). More males (9.6%) than females (3.2%) of the iodine-131 exposed group developed FTA and FTC. In the unirradiated control group thyroid lesions were observed in 4/53 females and 1/61 male mice ($p = 0.18$). Gender differences were even lower in the

Table 4. Thyroid lesions in the different F1 hybrid mice.

hybrid	thyroid lesion[a]		NAD	SH	CH	FTA	FTC
C3B6F1[b]	control[c] (n = 82)	female	36 (43.9%)	3 (3.7%)	0	1 (1.2%)	0
		male	41 (50.0%)	1 (1.2%)	0	0	0
	I-131[d] (n = 59)	female	7 (11.9%)	7 (11.9%)	15 (25.4%)	1 (1.7%)	0
		male	11 (18.6%)	12 (20.3%)	4 (6.8%)	1 (1.7%)	1 (1.7%)
CC3F1[b]	control[c] (n = 9)	female	4 (44.4%)	0	0	0	0
		male	5 (55.6%)	0	0	0	0
	I-131[d] (n = 24)	female	3 (12.5%)	7 (29.2%)	0	0	0
		male	5 (20.8%)	6 (25.0%)	0	2 (8.3%)	1 (4.2%)
JFC3F1[b]	control[c] (n = 23)	female	9 (39.1%)	0	0	0	0
		male	14 (60.9%)	0	0	0	0
	I-131[d] (n = 31)	female	18 (58.1%)	1 (3.2%)	2 (6.5%)	0	1 (3.2%)
		male	8 (25.8%)	1 (3.2%)	0	0	0

[a] abbreviations: NAD - no abnormality detected, SH - simple hyperplasia, CH - complex hyperplasia, FTA - follicular thyroid adenoma, FTC - follicular thyroid carcinoma

[b] F1 hybrids: C3B6F1 – C3H × C57BL/6, CC3F1 – BALB/c × C3H, JFC3F1 – JF1 × C3H

[c] unirradiated control mice

[d] mice perinatally exposed to iodine-131

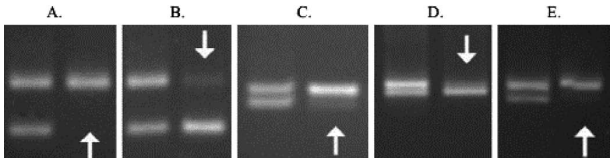

Fig. 4. Microsatellite analysis. Representative images of ethidiumbromide stained agarose gels with PCR products from C3H x C57BL/6 F1 hybrid mice are shown. PCR products from heterozygous normal tissue (tail tip) (left side) are compared to PCR products from thyroid lesions (right side). **A.** D14Mit125; complex hyperplasia. **B.** D14Mit125; follicular adenoma. **C.** D5Mit345; follicular adenoma. **D.** D4Mit166; follicular adenoma. **E.** D5Mit349; follicular adenoma. **B.** and **D.** show a deletion of the longer allele. **A.**, **C.** and **E.** show a loss of the shorter allele.

group of I-131-exposed mice, 34/62 females and 27/52 males showed thyroid lesions versus 28 females and 25 males with normal thyroids ($p = 0.85$). Only in C3B6F1 hybrid females was there a significantly ($p < 0.005$) higher number of complex hyperplasias compared to males.

Sequencing of mutational hotspot regions of different cancer-related genes

Several genes are known to be mutated in human thyroid cancers, including the proto-oncogene *RET,* and the oncogenes *BRAF, HRAS, KRAS, KIT* and the tumour suppressor gene *TP53.* The mutational hotspots described for the human genes were sequenced in the homologous mouse genes in 47 I-131-exposed mice (3 normal thyroids, 31 hyperplasia, 9 FTA, 4 FTC) and 4 unirradiated controls (2 hyperplasia, 1 FTA, 1FTC).

The *Braf* hotspots indicated by the known human thyroid cancer hotspots[26] were studies in the mouse gene and checked for the presence of the analogon to the human V600E mutation. Other putative *Braf* hotspots, namely exons 14 and 18, were also examined, with negative findings. For *Hras* and *Kras* codons 12, 13 and 61 were sequenced and analysed. No mutational base changes were found in these hotspot codons. The oncogene *Kit* was sequenced at the hotspots in murine exons 11 and 17, homologous to human codons 553–561, 816 and 820. Subsequently the tumour suppressor gene *Trp53* hotspot regions in murine exons 5, 7 and 8 were sequenced and analysed for mutations and polymorphisms without findings. Exons 10 and 11 of the *Ret* proto-oncogene showed no mutations. In conclusion, no germline mutations were found, but various silent polymorphisms were detected, depending on the strain background.

Microsatellite analysis of allelic imbalance

Allelic imbalance at 14 microsatellite loci was found in CH and FTA from radioiodine-exposed C3B6F1 mice (Fig. 4). At the D14Mit125 locus loss of one allele was detected in 5 C3B6F1 thyroid tissue samples, 4 (33%) CH and 1 (25%) FTA (Table 5). Other markers showed allelic imbalance only in FTA cases. No allelic imbalance was found in the FTA sample from the C3B6F1 control mouse and in I-131-exposed CC3F1 mice. A significantly higher incidence of allelic imbalance and loss of heterozygosity per locus was found in CH and FTA tissue samples, compared to thyroid tissue showing no pathological findings (NAD) or SH ($p < 0.05$).

Table 5. 14 different markers showed LOH in the DNA of thyroid samples from C3B6F1 mice.

Candidate Genes	Microsatellite Marker	NAD and SH[a] (I-131) (n = 18)	CH[a] (I-131) (n = 12)	FTA[a] (I-131) (n = 4)	FTA[a] (unirr.) (n = 1)
Cdkn2a	D4Mit166	0	0	1 (25%)	0
	D5Mit193	0	0	1 (25%)	0
	D5Mit345	0	0	1 (25%)	0
	D5Mit349	0	0	1 (25%)	0
	D5Mit13	0	0	2 (50%)	0
	D5Mit352	0	0	2 (50%)	0
Ret, Braf, Met	D6Mit1	0	0	1 (25%)	0
	D6Mit243	0	0	1 (25%)	0
Trp53	D11Mit38	0	0	1 (25%)	0
Rb1	D14Mit125	0	4 (33%)	1 (25%)	0
	D14Mit225	0	0	1 (25%)	0
	D14Mit215	0	0	1 (25%)	0
Pten	D19Mit19	0	0	1 (25%)	0
	D19Mit41	0	0	1 (25%)	0

[a] abbreviations: NAD - no abnormality detected, SH - simple hyperplasia, CH - complex hyperplasia, FTA - follicular thyroid adenoma, FTC - follicular thyroid carcinoma

DISCUSSION

After the Chernobyl nuclear accident an increase in thyroid malignancies was observed, especially in children and adolescents living in the areas of radioactive fallout. However, not all children exposed to the same dose of radioiodine develop thyroid lesions. In addition to unknown stochastic effects, predisposing genetic effects may exist that put some individuals at higher risk than others. To determine if there is an influence of the genetic background on the incidence of thyroid lesions, we used a mouse model to induce

thyroid lesions by I-131 exposure in a series of F1 hybrid mouse crosses. During the follow-up period of 18 months the I-131-exposed mice but not non-irradiated control mice developed thyroid lesions at a high rate resembling the post-Chernobyl situation in man.[5)]

The different mouse hybrids all reacted to the irradiation with a significantly higher occurrence of thyroid lesions compared to the non-irradiated control groups. However, the incidence of total thyroid lesions was significantly lower in the radiation-exposed JFC3F1 hybrids compared to either the C3B6F1 or CC3F1 radioiodine-exposed mice. In this study we used mouse hybrids where one parent was from the inbred C3H strain, so that one half of the genetic background of each F1 hybrid always comes from C3H. The other half originates from either JF1, C57BL/6 or BALB/c. The differences in sensitivity we see lead us to the conclusion that the genetic contribution from JF1 mice is sufficient to suppress the development of thyroid lesions after I-131 exposure. JF1 mice (Japanese Fancy Mouse 1) originate from Japanese wild mice, *M. m. molossinus*,[27)] and show a high degree of polymorphisms compared to common laboratory mice.[28)] Therefore JF1 mice are assumed to show a greater genetic distance from the other strains that can result in the manifestation of altered phenotypes, e.g. a higher resistance to radiation.

Despite the different frequency of thyroid lesions in JFC3F1 and C3B6F1 mice, these hybrids showed an equal distribution of simple and complex hyperplasias. In contrast, CC3F1 hybrid mice showed a high frequency of simple hyperplasia but the progression to complex hyperplastic disease was absent, pointing to a specific suppression of progression to CH in BALB/c hybrids. The absence of cases of CH may indicate the independent development of complex thyroid lesions and makes it unlikely that murine thyroid lesions develop from progression of simple to complex hyperplasia and further to adenoma and carcinoma.[14–16)] On the other hand about half of the FTCs detected in our hybrid mice were accompanied by hyperplasia that points to the development of carcinoma from simple and more complex disease entities.

No papillary thyroid carcinoma was found in our mice. In contrast, the thyroid carcinomas in human patients that are reported after radiation exposure, particularly in Chernobyl-associated cases, showed a mainly papillary growth pattern, diagnosed by presence of typical nuclear features, such as ground glass nuclei, nuclear grooves, pseudoinclusions and large overlapping nuclei. A high prevalence of *RET/PTC* rearrangements that activate the RET gene in the thyroid were documented in post-Chernobyl PTC, the most frequent are *RET/PTC1* and *RET/PTC3*.[29)] A correlation of nuclear morphology with *RET* activation (absent in our animals) was suggested.[30)] The existence of murine PTC was previously reported in transgenic mice,[31)] and also in I-131-induced thyroid carcinomas in 2-year-old CBA mice.[24)] In our study no thyroid carcinoma unequivocally presented the morphological nuclear criteria needed for classification as PTC. This might be due to the different mouse strains we evaluated in our study, the age of the mice at sacrifice, or a result of different classification criteria.

The discrepancies between the comparative pathologies of mouse and human thyroid lesions are influenced by the different approaches used to categorise the lesions. Classification schemes used in mouse pathology tend to use more general histological aspects of benign and malignant lesions applicable for each epithelial tumour, like mitosis, invasion and size of the tumour.[16)] In contrast, the definitions of types and subtypes for human thyroid lesions are more precise and include knowledge about prognostic and predictive characteristics. Using the human WHO classification criteria in the mouse shifts the diagnostic criteria in favour of benignity. Several complex hyperplasias could have be graded as adenomas when using the murine classification scheme and applying the increased architectural and cytological complexity and disregarding the point that human FTA predominantly show solitary nodules. In human pathology the classification "benign tumour" (adenoma) specifies a neoplasm that rarely or never progress to carcinoma. Human hyperplasias (endemic and sporadic goiter) are regarded as reversible changes of the thyroid gland that appear in response to iodine deficiency and bear a minimal risk for progression to malignancies. In contrast, microscopic foci with papillary nuclear features and *RET* rearrangements were found in human thyroid nodules with incomplete morphological signs of PTC, suggesting that these foci may be the precursors of papillary cancer.[32)] Experimental thyroid tumours in mouse suggest that there is typically a progression from hyperplasia through adenoma to carcinoma. Thus, hyperplasias are considered as important preneoplastic lesions in mice.[14–16,33)] This view is supported by the simultaneous appearance of SH, CH, FTA and FTC in the same thyroid gland in our study. Altogether the human classification criteria recommended by the WHO are readily applicable for the classification of murine thyroid lesions. The use of a common frame of reference in mouse and man supports a better comparability of human and mouse pathology of thyroid lesions.

Genetic changes are a critical feature in the development of malignancies. We examined the thyroid tissue from radioiodine-exposed mouse hybrids for changes in genes frequently mutated in human thyroid tumours and sequenced the hotspot regions of genes, such as *Ret, Braf, Hras, Kras, Kit* and *Trp53*. However, we did not find mutations in the hotspots of any of these genes in the murine thyroid lesions. *BRAF* mutations are common in human PTC, but are not frequently seen in post-Chernobyl childhood PTC and other radiation associated thyroid carcinomas.[21,34,35)] Interestingly, no germline mutations were found in the coding regions of *HRAS, KRAS, NRAS, BRAF, MEK1 and MEK2* of 24 indi-

viduals with FNMTC who had developed PTC.[36)]

Microsatellite analysis of allelic imbalance showed no alterations in a single adenoma of an unirradiated mouse nor in radioiodine-exposed normal thyroids and SH. However, 33% of CH in irradiated C3B6F1 mice had lost one allele at the D11Mit125 locus and 25 to 50% of FTA showed a loss of one allele occuring at 14 different loci on 6 chromosomes, indicating the accumulation of alterations in more severe thyroid lesions and suggesting indeed that CH is a progression from SH. The radiation associated FTAs were characterised by the loss of large regions where two or more adjacent markers show LOH. Studies on human radiation-associated thyroid carcinomas are in accordance with these results as they also showed no or very low rate of allelic imbalance in the control groups, even in normal thyroids that received radiation treatment.[37,38)] In these studies, as in our results, instability of at least one microsatellite marker was detected in 31 to 50% of radiation associated thyroid tumours, while benign tumours showed a lower frequency than malignant tumours.[37,38)]

In conclusion we have been able to demonstrate the existence of strain-specific genetic susceptibility to radioiodine-induced murine thyroid tumours. Significant differences in the frequency of thyroid lesions were determined in different hybrid mouse crosses from inbred strains. Typically mutated genes in thyroid cancer are not obviously involved in murine radioiodine-induced thyroid tumours.

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