0021-972X/01/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 86, No. 7
Printed in U.S.A.

RET/PTC Rearrangements in Thyroid Nodules: Studies in Irradiated and Not Irradiated, Malignant and Benign Thyroid Lesions in Children and Adults*

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ABSTRACT

Rearrangements of the RET proto-oncogene may occur in both naturally occurring and radiation-induced papillary thyroid carcinomas. Conflicting results on the frequency and type of RET/PTC rearrangements have been reported in relation to age, radiation exposure, and histological tumor variant.

We designed the present study to evaluate in a single laboratory, using the same methodologies, the pattern of RET/PTC activation in thyroid tumors from different groups of patients (exposed or not exposed to radiation, children or adults, with benign or malignant tumors) in relationship to the above mentioned variables.

We studied 154 patients with benign nodules (n = 65) or papillary thyroid cancer (n = 89). In the last group, 25 were Belarus children exposed to the post-Chernobyl radioactive fallout, 17 were Italian adults exposed to external radiotherapy for benign diseases, and 47 were Italian subjects (25 children and 22 adults) with no history of radiation exposure. Among patients with benign thyroid nodules, 21 were Belarus subjects (18 children and 3 adults) exposed to the post-Chernobyl radioactive fallout, 8 were Italian adults exposed to external radiation on the head and neck, and 36 were Italian adults with naturally occurring benign nodules.

The overall frequency of RET/PTC rearrangements in papillary thyroid cancer was 55%. The highest frequency was found in post-Chernobyl children and was significantly higher ($P = 0.02$) than that found in Italian children not exposed to radiation, but not significantly higher than that found in adults exposed to external radiation. No difference of RET/PTC rearrangements was found between samples from irradiated (external x-ray) or not irradiated adult patients, as well as between children and adults with naturally occurring, not irradiated, thyroid cancer.

When analyzing the type of RET/PTC rearrangement (RET/PTC1 or RET/PTC3), no major difference was apparent. In addition, eight cases with an unknown RET/PTC rearrangement and three cases with the concomitant expression of RET/PTC1 and RET/PTC3 were found. No significant correlation was observed between the frequency and/or the type of RET/PTC rearrangement and clinical-epidemiological features of the patients such as age at diagnosis, age at exposure, histological variant, gender and tumor-node-metastasis (TNM) categories.

RET/PTC rearrangements were also found in 52.4% of post-Chernobyl benign nodules, in 37.5% of benign nodules exposed to external radiation and in 13.9% of naturally occurring nodules ($P = 0.005$, between benign post-Chernobyl nodules and naturally occurring nodules). The relative frequency of RET/PTC1 and RET/PTC3 in rearranged benign tumors showed no major difference.

In conclusion, our results indicate that the presence of RET/PTC rearrangements in thyroid tumors is not restricted to the malignant phenotype, is not higher in radiation-induced tumors compared with those naturally occurring, is not different after exposure to radioiodine or external radiation, and is not dependent from young age. Other factors, probably influenced by ethnic or genetic background, may act independently from or in cooperation with radiation, to trigger the DNA damage leading to RET proto-oncogene activation. (*J Clin Endocrinol Metab* **86:** 3211–3216, 2001)

REARRANGEMENTS OF THE RET proto-oncogene, mainly in the form of RET/PTC1 and RET/PTC3, are found in papillary thyroid carcinomas and have been shown to play a pathogenic role (1–3). Several types of rearrangements have been described according to the activating genes involved in the rearrangement with the RET gene (4–10) with frequencies varying widely among different countries and different age groups (11–12). Few and controversial data have been reported on the presence of RET/PTC rearrangements in benign thyroid tumors (13–14).

Ionizing radiation, the only recognized etiological factor in the pathogenesis of papillary thyroid cancer both after external irradiation (15, 16) and after the Chernobyl nuclear disaster in 1986 (17–19), is responsible for the generation of RET/PTC rearrangements, as supported by *in vitro* studies (20) and by recent studies in post-Chernobyl thyroid tumors (21, 22).

Conflicting results on the frequency and type of RET/PTC rearrangements in relation to age, radiation exposure, and histological tumor variant have been reported in different series (23–27). Because the above data were obtained by several groups, using different experimental techniques, we designed the present study to evaluate in a single laboratory, using the same methodologies, the pattern of RET/PTC activation in thyroid tumors in relationship to different factors, such as radiation exposure (irradiated *vs.* not irradiated), type of radiation (internal *vs.* external), age (children and

Received December 27, 2000. Revision received March 9, 2001. Accepted March 27, 2001.

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* Supported in part by a grant from European Communities: INCO-Copernicus Project (IC15-CT980314) and MURST (ex 40%) 2000.

TABLE 1. Epidemiological data of patients with papillary thyroid cancer and benign neoplasm included in the study

	Study groups	Radiation exposure	F/M	Age at diagnosis		Age at exposure	
				Median (yr)	Range (yr)	Median (yr)	Range (months-yr)
Papillary							
Belarus	Children (n = 25)	Radioiodine	11/14	12.0	10–17	1.0	In uterus–7 yr[a]
Italian	Children (n = 25)	None	16/9	16.0	8–18		
Italian	Adults (n = 17)	External irradiated	12/5	38.0	18–70	6.0	2 months–47 yr
Italian	Adults (n = 22)	None	14/8	40.5	19–72		
Benign							
Belarus	Children/adults (n = 18/3)	Radioiodine	15/6	14.5	8–57	5.0	12 months–47 yr
Italian	Adults (n = 8)	External irradiated	5/3	35.0	23–71	9.0	8 months–44 yr
Italian	Adults (n = 36)	None	28/8	46.0	19–76		

[a] Six subjects were exposed in uterus (1st month, 4th month (n = 2), 7th month, 8th month, and 9th month of pregnancy).

adolescents *vs.* adults) and pathology (benign *vs.* malignant tumors).

Patients and Methods

Patients

We studied 154 patients submitted to surgical treatment for benign (n = 65) or malignant (n = 89) thyroid nodules. As indicated in Table 1, all malignant nodules were from patients with papillary thyroid cancer: 25 were Belarus children exposed to the post-Chernobyl radioactive fallout, 17 were Italian adults exposed to external radiotherapy for benign diseases mostly during childhood, and 47 were Italian subjects (25 children and 22 adults) with no history of radiation exposure. Age at diagnosis was comparable in the two groups of childhood thyroid cancer and in the two groups of adults. Age at radiation exposure was also similar in most of the Belarus children compared with adult subjects who received external irradiation during childhood, with the exception of six Belarus children who were exposed in uterus and of three Italian patients who received radiation therapy when adults (24, 35, and 47 yr). As expected, the latency period between radiation exposure and clinical manifestation of the tumor was much shorter in Belarus children.

Among patients with benign thyroid nodules after radiation exposure, 21 were Belarus subjects (18 children and 3 adults) exposed to the post-Chernobyl radioactive fallout and 8 were Italian adults exposed to external radiation. Age at exposure in these two groups was not comparable. In fact, 17 of the 21 Belarus subjects, as opposed to 3 of the 8 Italian subjects, had been exposed when less than 10 yr old. As a control group, we studied 36 Italian adult patients with naturally-occurring benign thyroid nodules.

Histology of all samples was reviewed in our Center to use uniform criteria of diagnosis. The pathological diagnosis of malignant and benign cases is reported in Table 2.

RNA isolation from thyroid tissues

Fresh and paraffin-embedded tissues were used in 84 and 70 cases, respectively. In case of fresh tissue, total RNA was extracted from 30–100 mg tissue using a commercial kit based on the guanidinium isothiocyanate method (RNAzol B Tel-Test, Friendswood, TX). When using paraffin-embedded tissues, total RNA was isolated from five sections (20 μm thick) immediately adjacent to the diagnostic slides. RNA extraction was performed as described previously (25).

RT

Total RNA was reverse transcribed into complementary DNA (cDNA) using Avian Mieloblastosis Virus reverse transcriptase. In particular, 50% (5 μL) of RNA recovered from paraffin-embedded tissues or 5 μg RNA recovered from fresh tissues were used for the production of cDNA in a mixture containing 10 mM $MgCl_2$, 1 mM dNTP, 0.45 U random hexameres (Amersham Pharmacia Biotech, Uppsala, Sweden), 23 U reverse transcriptase (Promega Corp., Madison, WI), and 80 U recombinant RNasin (Promega Corp.) in a final volume of 100 μL. After a 1-h incubation at 42 C, the enzyme was heat inactivated at 95 C for 5 min.

TABLE 2. Pathological features of papillary thyroid cancer and benign neoplasm included in the study

	Histological variants			
	Classical n (%)	Solid n (%)	Follicular n (%)	Others[a] n (%)
Papillary				
Belarus children (n = 25)	7 (28.0)	6 (24.0)	11 (44.0)	1 (4.0)
Italian children (n = 25)	12 (48.0)	0	11 (44.0)	2 (8.0)
Italian irradiated adults (n = 17)	10 (58.9)	1 (5.8)	5 (29.5)	1 (5.8)
Italian not irradiated adults (n = 22)	16 (72.8)	1 (4.5)	2 (9.0)	3 (13.7)

	Follicular adenomas n (%)	Hyperplastic nodules n (%)
Benign		
Post-Chernobyl cases (n = 21)[b]	14 (66.6)	7 (33.3)
Italian irradiated adults (n = 8)	2 (25.0)	6 (75.0)
Italian not irradiated adults (n = 36)	28 (77.8)	8 (22.2)

[a] Including sclerosing, Hürthle, and tall cell variants.
[b] Eighteen children and three adults.

PCR amplification and Southern blotting

The cDNA quality was tested by the amplification of a 236-bp sequence of c-N-ras (25). All samples included in the study demonstrated detectable levels of N-ras transcript. RET/PTC1 and RET/PTC3 were analyzed in all samples by PCR using primers reported previously (25). In cases positive for RET/PTC1 and/or RET/PTC3, an internal control was introduced to role out the possibility of PCR contamination consisting of the use of a second pair of primers, annealing externally to the previous PCR product (RET/PTC1: ext-F 5′gtcgggggcattgtcatct3′, ext-R 5′aagttcttccgagggaattc3′; RET/PTC3: ext-F 5′tggcttatccaaaagcagac3′, ext-R 5′gccccatacaatttgatgac3′). Cases positive for both pairs of primers were considered as real positives. To identify types of RET rearrangements other than RET/PTC1 and RET/PTC3, negative samples for both rearrangements were studied for the expression of the tyrosine kinase (TK) and extracellular (EC) domains of the RET gene. All samples showing TK expression not associated to EC expression were considered positive for a RET rearrangement (RET/PTCX) different from RET/PTC1 and RET/PTC3 (25). PCR protocol was the same for each amplified fragment: ten microliters of cDNA were added to a mixture containing 1.5 mM $MgCl_2$, 20 pmol of each primer, 200 μM dNTP, and 0.3 U *Taq* (Promega Corp.) in a final volume of 30 μL. Thirty-five cycles of denaturation (94 C for 1 min), annealing (55 C for 1 min), and extension (72 C for 1.5 min) were conducted on an automated heat block (DNA thermal cycler; MJ).

Ten microliters of PCR product were electrophoresed in a 1.5% agarose gel and blotted onto a nylon membrane. Each filter was then hybridized with internal probes (Table 3) specific for each amplified fragment, labeled using a chemoluminescent method (Gene Images 3′-Oligolabelling and CDP-Star Detection System; Amersham Pharmacia Biotech).

TABLE 3. Primers and probes used for RET/PTC screening

	Size (bp)	Primer sequences (5′–3′)	Oligoprobes (5′–3′)
c-ras	236	For: ATGACTGAGTACAAACTGGT Rev: AGGAAGCCTTCGCCTGTCCT	CAAGTGGTTATAGATGGTGA
c-ret, TK	155	For: GGAGCCAGGGTCGGATTCCAGTTA Rev: CCGCTCAGGAGGAATCCCAGGATA	ACGCAAAGTGATGTATGGTCT
c-ret, EC	184	For: GGCGGCCCAAGTGTGCCGAACTT Rev: CCCAGGCCGCCACACTCCTCACA	GTAACAGTGGAGGGGTCATATG
RET/PTC1 (internal primers)	165	For: GCTGGAGACCTACAAACTGA Rev: GTTGCCTTGACCACTTTTC	GGCACTGCAGGAGGAGAACCGCGA
RET/PTC3 (internal primers)	242	For: AAGCAAACCTGCCAGTGG Rev: CTTTCAGCATCTTCACGG	GGTCGGTGCTGGGTATGTAAGGA
RET/PTC1 (external primers)	204	For: GTCGGGGGGCATTGTCATCT Rev: AAGTTCTTCCGAGGGAATTC	GGCACTGCAGGAGGAGAACCGCGA
RET/PTC3 (external primers)	278	For: TGGCTTATCCAAAAGCAGAC Rev: GCCCCATACAATTTGATGAC	CTTGGAGAACAGTCAGGAGGA

All primers and probes have been reported previously (25), with the exception of the external primer for RET/PTC1 and RET/PTC3. These primers were designed using the computerized program "primers 3 output." For, Forward; Rev, reverse.

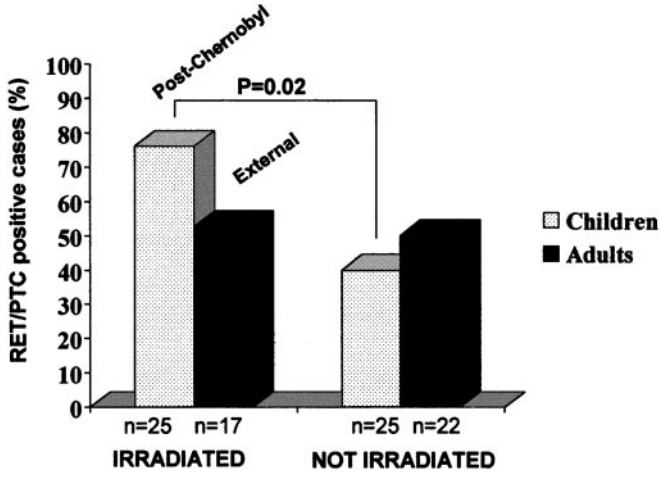

FIG. 1. Frequency of RET/PTC rearrangements in radiation-induced (post-Chernobyl and external irradiated) and naturally occurring papillary thyroid carcinomas, in children and adults. The post-Chernobyl cases are from Belarus, the others groups are from Italy.

Results

RET/PTC in papillary thyroid carcinomas

The frequency of RET/PTC rearrangements in papillary thyroid cancer (considering all subjects) was 55% (49 of 89). As shown in Fig. 1, the highest frequency was found in post-Chernobyl children (76%) and was significantly higher ($P = 0.02$, by X^2) when compared with the group of Italian children not exposed to radiation (40%) and when compared with all the others groups combined ($P = 0.02$, by χ^2). However, when the post-Chernobyl-positive cases were separately compared with the two groups of adults (exposed or not exposed to radiation) no significant difference was found. In particular, no difference was found between post-Chernobyl-positive cases and the group of adults exposed to external radiation (52.9%), between samples from irradiated (external x-ray) or not irradiated adult patients (52.9% *vs.* 50%, respectively), and between children and adults with naturally occurring, not irradiated, thyroid cancer (40.0% *vs.* 50%, respectively).

When analyzing the type of RET/PTC rearrangement (Table 4), no major difference was apparent. Compared with RET/PTC1, RET/PTC3 rearrangement was slightly more frequent in all groups but the one of naturally occurring childhood tumors. The occurrence of tumors characterized by the expression of the TK domain, without the EC domain of RET (RET/PTCX) was found in 8 cases (9.1%). Six of them were found among post-Chernobyl tumors including four of six children who were in uterus at the time of radiation exposure. Representative cases of RET/PTC rearrangements in post-Chernobyl tumors are shown in Fig. 2.

Three papillary thyroid carcinomas (two in post-Chernobyl children and one in an adult subject with naturally occurring tumor) showed the simultaneous presence of RET/PTC1 and RET/PTC3 rearrangements. The two rearrangements showed different levels of expression when compared with the expression of c-N-Ras (semiquantitative analysis), suggesting that each rearrangement was in a distinct subpopulation of neoplastic cells (polyclonal pattern).

No significant correlation was observed between the frequency and/or the type of RET/PTC rearrangement and clinical-epidemiological features of the patients such as age at diagnosis, age at exposure, histological variant, gender and tumor-node-metastasis (TNM) categories.

RET/PTC in benign thyroid nodules

RET/PTC rearrangements were found in 29.2% (19 of 65) of benign nodules. As shown in Fig. 3, RET/PTC rearrangements were found in 52.4% of post-Chernobyl benign nodules, in 37.5% of benign nodules exposed to external radiation and in 13.9% of naturally occurring nodules. The difference between benign post-Chernobyl nodules and naturally occurring nodules was significant ($P = 0.005$ by X^2), whereas no difference was found between external irradiated nodules and naturally occurring nodules. This last result may be due to the quite small number (n = 8) of patients with benign nodules exposed to external radiation.

The relative frequency of RET/PTC1 and RET/PTC3 in rearranged tumors showed no major difference (Table 5). A few cases of RET/PTCX were found in benign nodules both exposed and not exposed to external radiation. Two cases of simultaneous expression of RET/PTC1 and RET/PTC3 were found in post-Chernobyl benign nodules.

TABLE 4. RET/PTC rearrangements in irradiated (post-Chernobyl Belarus children and Italian adults exposed to external radiations) and naturally occurring (Italian children and adults) papillary thyroid carcinomas

	RET/PTC-positive cases					
	PTC1 n (%)	PTC3 n (%)	PTC1+PTC3 n (%)	PTCX n (%)	All n (%)	*P*
Irradiated						
Post-Chernobyl children (n = 25)	4 (16.0)[a]	7 (28.0)	2 (8.0)	6 (24.0)	19 (76.0)	0.02
Italian adults (n = 17)	4 (23.5)	5 (29.4)	0	0	9 (52.9)	
Naturally occurring						
Italian children (n = 25)	5 (20.0)	4 (16.0)	0	1 (4.0)	10 (40.0)	
Italian adults (n = 22)	2 (9.0)	7 (31.8)	1 (4.5)	1 (4.5)	11 (50.0)	
Total (n = 89)	15 (16.8)	23 (25.8)	3 (3.3)	8 (9.0)	49 (55.0)	

[a] One case is a variant of PTC1(PTC1 long) reported by Elisei *et al.* (42).

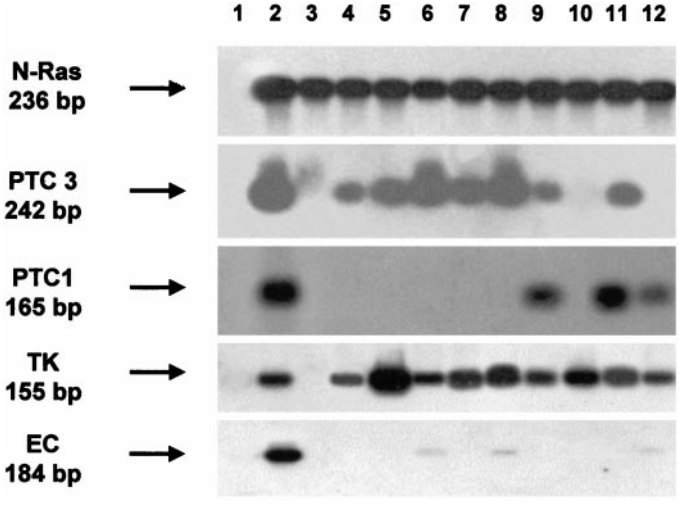

FIG. 2. Representative cases of RET/PTC rearrangements in post-Chernobyl papillary thyroid tumors (lanes 3–12) and negative and positive controls (lanes 1 and 2); the samples in lanes 4–8 are rearranged only for RET/PTC3, sample 12 only for RET/PTC1, 9 and 11 for both RET/PTC1 and RET/PTC3. Sample 10 (RET/PTCX), expressing the TK domain but not the EC fragment of RET, is neither RET/PTC1 and RET/PTC3.

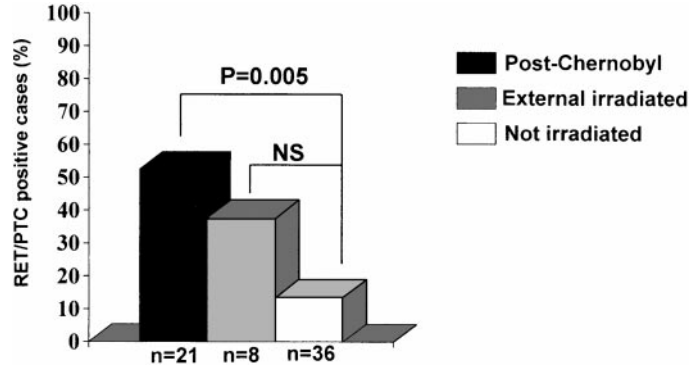

FIG. 3. Frequency of RET/PTC rearrangements in irradiated (post-Chernobyl and external irradiated) and not irradiated benign thyroid neoplasm. The post-Chernobyl cases are from Belarus, the others groups are from Italy.

Discussion

In this study, we analyzed the frequency and type of RET/PTC rearrangements in a series of irradiated or not irradiated benign (n = 65) and malignant (n = 89) thyroid nodules in relation to the type of irradiation (external *vs.* internal), age at diagnosis, histology and gender. One by one, these variables have been analyzed in previous reports. The present study differs from the others because, for the first time, all the patients categories have been investigated in the same laboratory, using the same methodology.

According to the current opinion based on the available literature, RET/PTC rearrangements seem to be related to papillary thyroid carcinoma (28), radiation exposure (13, 23–25), and young age (26).

This assumption is not supported by the present study; in fact, in our series RET/PTC rearrangements were not restricted to papillary thyroid cancer. A relatively high frequency of RET/PTC rearrangements was also found in benign nodular thyroid diseases of patients exposed to post-Chernobyl fallout (52.4%) or external radiation (37.5%), and, albeit less frequently, in patients with benign nodules not exposed to radiation (13.9%). While no RET/PTC rearrangements in benign thyroid lesions have been found by Santoro *et al.* (28) and Thomas *et al.* (27), RET mutations have been found by Bounacer *et al.* (13) in irradiated benign thyroid nodules of French patients and by Ishizaka *et al.* (14) in sporadic follicular adenomas. Among other possibilities, the occurrence of RET/PTC rearrangements in benign nodules might be explained by the presence of microfoci of papillary thyroid carcinoma, or even of few neoplastic cells within the benign tumor, not easily detectable by histology. This possibility is in keeping with the high frequency of RET/PTC rearrangements found in papillary microcarcinomas: 42.3% in an Italian series (29) and 77% in a Canadian series (30). An alternative explanation is that RET/PTC rearrangement is an early genetic event favoring the transition from benign to malignant phenotype.

In the present study, the effect of post-Chernobyl and external radiation has been compared in children and adults with benign and malignant thyroid lesions. No significant differences have been found in the frequency of RET/PTC rearrangements in both malignant and benign tumors of our series, indicating that these changes are not related to the modality of irradiation. When radiation-induced and naturally occurring thyroid carcinomas have been compared in adults and children, a significantly higher frequency of RET/PTC rearrangements has been only found in Belarus children with respect to Italian children. At variance with our data, no

TABLE 5. RET/PTC rearrangements in irradiated and naturally occurring benign nodular thyroid disease

	RET/PTC-positive cases				
	PTC1 n (%)	PTC3 n (%)	PTC1+PTC3 n (%)	PTCX n (%)	All n (%)
Irradiated					
Post-Chernobyl[a] (n = 21)	3 (14.3)	6 (28.6)	2 (9.5)	0	11 (52.4)
Italian adults (n = 8)	0	0	0	3 (37.5)	3 (37.5)
Naturally occurring					
Italian adults (n = 36)	1 (2.7)	1 (2.7)	0	3 (8.3)	5 (13.9)
Total (n = 65)	4 (6.1)	7 (10.8)	2 (3.0)	6 (9.2)	19 (29.2)

[a] Eighteen children and three adults at the moment of the nuclear accident.

difference has been found by Nikiforov *et al.* (25) when comparing the frequency of RET/PTC rearrangements in Belarus children with post-Chernobyl cancer and naturally occurring American childhood tumors. The question of whether this difference could be due to different ethnic background is difficult to solve because the lack of the appropriate control (*i.e.* Belarus children with naturally occurring papillary thyroid carcinoma) in both studies, prevents the possibility to answer this question. It is worth noting that reported frequencies of RET/PTC rearrangements in sporadic papillary thyroid cancer vary widely among different countries, ranging from as low as 2.5% in Saudi Arabia to 59% in the United Kingdom (31–39).

Age has been advocated as a factor favoring the development of RET/PTC rearrangements. In this respect, it is of interest that when adults and children with naturally occurring papillary thyroid cancer were compared within the same country no significant difference in the frequency of RET/PTC rearrangements has been found in United Kingdom (36) and Japan (40) series as well as in the present study. Moreover, no difference has been reported by Smida *et al.* (41) between Belarus children and adults with post-Chernobyl thyroid cancer. All together, these data strongly suggest that age does not play a major role in the occurrence of RET/PTC-positive papillary thyroid cancer and indirectly support the relevance of the ethnic role.

A relative higher frequency of RET/PTC3 with respect to RET/PTC1 has been reported in post-Chernobyl thyroid cancer in earlier studies from this (23) and other laboratories (24, 25). In the present study the frequency of RET/PTC3 rearrangements was not different from that of RET/PTC1 in benign and malignant tumors of both children and adults, irrespective of whether irradiated or not irradiated. However, when cumulating the solid and follicular histological variants a prevalence of RET/PTC3 was found in post-Chernobyl tumors.

As in other series (38, 39), we found the concomitant expression of both RET/PTC1 and RET/PTC3 in the same tissue in a significant proportion of cases. Among other possibilities, this finding can be explained by the occurrence of radiation-induced paracentric inversion in both chromosomes 10 of a single cell giving rise to RET/PTC1 in one chromosome and RET/PTC3 in the other one. Alternatively radiations may induce RET/PTC1 and RET/PTC3 tumors in the same gland resulting in a polyclonal thyroid neoplasm. Based on the different quantitative expression of the two rearrangements in the same tumor, the last possibility might be favored. However, the differential expression of RET/PTC1 and RET/PTC3 can also be explained by chromosome 10 inversion in a single cell when RET/PTC1 and RET/PTC3 transcription is driven by different promoters, H4 and ELE1 respectively, with different activities.

As in other series (24, 25) we found several cases of RET/PTCX. The precise nature of these changes requires further studies, but it is interesting to note that in the present series most of the cases of RET/PTCX were found in thyroid tumors of children exposed while in uterus.

In conclusion, our results indicate that the presence of RET/PTC rearrangements in thyroid tumors is not restricted to the malignant phenotype, is not higher in radiation-induced tumors compared with those naturally occurring, is not different after exposure to radioiodine or external radiation and is not dependent from young age. Other factors, probably influenced by ethnic or genetic background, may act independently from or in cooperation with radiations, to trigger the DNA damage leading to RET proto-oncogene activation.

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