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Dose-Dependent Generation of *RET/PTC* in Human Thyroid Cells after *in Vitro* Exposure to γ-Radiation: A Model of Carcinogenic Chromosomal Rearrangement Induced by Ionizing Radiation

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Ionizing radiation is a well-known risk factor for thyroid cancer in human populations. Chromosomal rearrangements involving the *RET* gene, known as *RET/PTC*, are prevalent in thyroid papillary carcinomas from patients with radiation history. We studied the generation of *RET/PTC* in HTori-3 immortalized human thyroid cells exposed to a range of doses of γ-radiation and harvested 2, 5–6, and 9 d later. *RET/PTC1* and *RET/PTC3* were detected by RT-PCR followed by Southern blotting and hybridization with internal oligonucleotide probes. No *RET/PTC* was found in cells harvested 2 and 5–6 d after irradiation, whereas 59 *RET/PTC* events were detected in cells collected 9 d after exposure. The average rate of *RET/PTC* induction was 0.1×10^{-6} after exposure to 0.1 Gy, 1.6×10^{-6} after 1 Gy, 3.0×10^{-6} after 5 Gy, and 0.9×10^{-6} after 10 Gy. When adjusted for cell survival, the rate after 10 Gy was comparable with those after 5 Gy. *RET/PTC1* was more common than *RET/PTC3* after each dose, comprising 80% of all rearrangements. In this study, we demonstrate a dose-dependent induction of *RET/PTC* rearrangements in human thyroid cells after exposure to 0.1–10 Gy γ-radiation. This provides additional evidence for a direct link between this genetic event and radiation exposure and offers a powerful experimental system for studying radiation-induced carcinogenesis in the thyroid gland. (*J Clin Endocrinol Metab* 90: 2364–2369, 2005)

EXPOSURE TO IONIZING radiation is a well-established risk factor for thyroid cancer. The association was first suggested more than 50 yr ago in children who received x-ray therapy in infancy for an enlarged thymus (1). During the following decades, numerous reports have documented an increased incidence of thyroid neoplasms in children after external radiation for different benign conditions of the head, neck, and thorax (2). An increased risk of thyroid cancer has also been linked to environmental irradiation and documented in survivors of atomic bomb explosions in Japan in 1945 (3), in residents of the Marshall Islands exposed to radioactive fallout in 1954 (4), and more recently in children exposed to radiation after the Chernobyl accident (5–7). The accident released unprecedented amounts of radioactive materials into the atmosphere (8). More than 10 million people were exposed to significant levels of radiation, and the thyroid dose was 3–10 times higher in children than in adults. In the most contaminated areas of southern Belarus, estimated thyroid doses in children were among the highest, ranging between less than 0.05 and 4 Gy (8). An increase in thyroid cancer incidence in children was noted in Belarus as early as 4 yr after the accident and reached almost 30-fold (100-fold in most contaminated Gomel region) in 1995 compared with those before Chernobyl and in other countries (5, 6). The risk of thyroid cancer correlated with proximity to Chernobyl and thyroid dose and in some areas was found to be linear in the dose interval 0.07–1.2 Gy (9).

Over the last few decades, significant progress has been achieved in the understanding of the biological mechanisms of radiation carcinogenesis. It has been shown that damage to cellular DNA is primarily responsible for mutagenesis and carcinogenesis and that double-strand breaks appear to be most important in the direct generation of mutations (10, 11). Incorrect end-joining of breaks causes mutations immediately, but it can also lead to genomic instability caused by cycles of chromatid fusion and breakage. More recently, evidence has emerged that, in addition to mutations arising directly from misrepaired DNA damage, genetic consequences may be related to the persistence of genomic instability or may affect cells in proximity to those exposed to radiation (bystander effect) (12, 13).

Relatively limited information, however, is available on the specific genetic alterations underlying cell transformation after radiation exposure. In this respect, thyroid cancer in children after the Chernobyl accident represents the most thoroughly studied population. In contrast to sporadic papillary carcinomas, which commonly harbor *BRAF* point mutations (14–16), post-Chernobyl tumors have a high prevalence of chromosomal rearrangements involving the *RET* gene, known as *RET/PTC*. Of several types of *RET/PTC* described, *RET/PTC1* and *RET/PTC3* are the most common, comprising more than 90% of all rearrangements found in sporadic and radiation-induced tumors. *RET/PTC1* is formed by *RET* fusion to the *H4* (also known as *D10S170*) gene (17, 18) and *RET/PTC3* by fusion to the *ELE1* (*RFG* or

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Abbreviations: FISH, Fluorescence *in situ* hybridization; NIS, sodium/iodide symporter; Tg, thyroglobulin.

ARA70) gene (19, 20). *RET/PTC1* and *RET/PTC3* are paracentric inversions, inv(10)(q11.2;q21) and inv(10)(q11.2), respectively, because both genes participating in each rearrangement are located on chromosome 10q (21). In contrast, *RET/PTC2* and other rare forms of *RET/PTC* are translocations resulting from the fusion of the intracellular domain of *RET* to heterologous genes located on different chromosomes (reviewed in Refs. 22 and 23).

The prevalence of *RET/PTC* rearrangement is approximately 20–30% in sporadic thyroid papillary carcinomas and is significantly higher in tumors from patients exposed to radiation. Indeed, in children exposed to radiation after the Chernobyl accident, *RET/PTC* was detected in 57–87% of tumors removed 5–8 yr after exposure and in 49–65% of tumors diagnosed 7–11 yr after the accident (24–29). A high prevalence of *RET/PTC* was also found in patients subjected to therapeutic x-ray irradiation for benign or malignant conditions (30, 31), providing additional evidence for the causal association between radiation exposure and generation of this rearrangement.

The occurrence of *RET/PTC* rearrangement has been studied in thyroid cells after *in vitro* radiation. *RET/PTC* was reported after high-dose radiation to human undifferentiated thyroid carcinoma cells (32) and to fetal human thyroid tissues transplanted into SCID mice (33, 34). In both studies, the rearrangement was detected by RT-PCR as early as 2 d after exposure to 50 Gy, a dose that is lethal for dividing cells. In addition, they employed either grafted fragments of fetal thyroid tissue, which are poorly amendable for experimental manipulations, or highly transformed, undifferentiated cells that are more susceptible to develop secondary genetic defects.

In this study, we aimed to establish and characterize a well-controlled model of induction of *RET/PTC* rearrangements in differentiated human thyroid cells after exposure to γ-radiation. This was achieved by subjecting HTori-3 cells to a range of doses implicated in the development of thyroid cancer in human populations.

Materials and Methods

Cell line and culture conditions

The experiments were performed on HTori-3 cells, which are human thyroid epithelial cells transfected with an origin-defective SV40 genome (35). They have been characterized as immortalized, partially transformed, differentiated cells that preserved the iodine-trapping ability and thyroglobulin (Tg) production. The cells were purchased from the European Tissue Culture Collection and grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum.

Chromosome analysis and fluorescence in situ hybridization (FISH)

Chromosomes were prepared and G-banded by standard procedures (36). Twenty-five metaphases were counted. For FISH, cells were grown directly on glass slides, fixed, and subjected to hybridization as previously described (37). A 207-bp bacterial artificial chromosome clone (RPC11-351D16, CHORI), spanning the entire *RET* gene region, was used as a probe. Bacterial artificial chromosome DNA was extracted and labeled with SpectrumGreen-dUTP (Vysis Inc.; Downers Grove, IL) by nick translation. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Microscopy was performed with a Leica TCS 4D confocal laser scanning fluorescence microscope with digital image capture (Leica, Wetzlar, Germany).

Expression of Tg and sodium/iodide symporter (NIS)

Expression of the *Tg* and *NIS* genes was tested by RT-PCR. Total RNA was extracted from HTori-3 cells and frozen normal thyroid tissue using Trizol reagent (Invitrogen). Three micrograms of total RNA were reverse transcribed in a volume of 20-μl reactions by using random hexamers priming and Superscript II RT (Invitrogen). The following exon-spanning primers were used: 5′-CCTCGCAGTTCAATCAGTCA-3′ (*Tg* forward), 5-TGGCTGAAGTAGCCTGAGGT-3′ (*Tg* reverse), 5′-CTCCCTGCTAACGACTCCAG-3′ (*NIS* forward), and 5′-GAGGTCCCACCACAACAATC-3′ (*NIS* reverse). For each PCR, 2 μl RT mixture was amplified in a final volume of 30 μl using 35 cycles of denaturation (94 C for 40 sec), annealing (59 C, 1 min for *NIS*; 57 C, 1 min for *Tg*), and extension (72 C for 1 min). cDNA from normal thyroid tissue was used as a positive control for gene expression. PCR products were electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining. The expected size of the PCR products was 207 bp for *NIS* and 303 bp for *Tg*. Amplification of a 247-bp sequence of the 3-phosphoglycerate kinase (*PGK*) gene was achieved as described elsewhere (38) and served as a control for the quantity and quality of the extracted RNA.

Cell irradiation

In most experiments, 1.5–1.8 $\times$ 10^6 cells were plated in a 75-cm^2 flask and 16 h later exposed to a single dose of γ-irradiation from a cesium-137 source at a dose rate of 1.7 Gy/min. A larger number of cells (up to 4 $\times$ 10^6) was used for higher doses to compensate for cell killing. Cells were exposed to 0, 0.1, 1, 5, and 10 Gy γ-irradiation, and at least four replicate plates were used per each dose. Four hours after irradiation, cells were split into six 12-well plates at a density of approximately 3 $\times$ 10^4 cells per well, grown for 2–9 d, and harvested. To sustain growth for 9 d, cells were transferred to 75-cm^2 flasks 4–5 d after seeding into 12-well plates.

Detection of RET/PTC rearrangements

RNA was extracted from each well or flask using a Trisol reagent (Invitrogen). Then, mRNA was purified using the Oligotex mRNA mini-kit (QIAGEN, Valencia, CA). RT-PCR was performed using a Superscript first strand synthesis system kit and random hexamer priming (QIAGEN). PCR was performed to detect *RET/PTC1* and *RET/PTC3* rearrangement simultaneously in one tube, using the following oligonucleotide primers: 5′-CAAGAGAACAAGGTGCTGAAG-3′ (*RET/PTC1* forward), 5′-CGGTATTGTAGCTGTCCCTTTC-3′ (*RET/PTC3* forward), and 5′-GCAGGTCTCGAAGCTCACTC-3′ (common reverse). PCR was carried out for 35 cycles. As a positive control and to test the sensitivity of the detection method, serial dilutions of the *RET/PTC1*-positive TPC-1 cells (39) and primary cultured papillary carcinoma cells from the *RET/PTC3* positive tumor in nonirradiated HTori-3 cells were used. Ten microliters of each PCR product was electrophoresed in a 1.5% agarose gel, transferred to the nylon membrane, and hybridized with ^{32}P-labeled oligonucleotide probes specific for *RET/PTC1* (5′-CGTTACCATCGAGGATCCAAA-3′) and *RET/PTC3* (5′-GAACAGTCAGGAGGTCCAA-3′) for 16 h at 42 C, washed, and exposed for autoradiography for 2–16 h. Evidence of *RET/PTC* rearrangement in the cells from a given flask was scored as one *RET/PTC* event.

Statistical analysis

The differences between the rate of *RET/PTC* induction after various doses were analyzed with one-way ANOVA, followed by *t* test for individual comparisons. The difference was considered statistically significant at $P < 0.05$.

Results

The originally established HTori-3 cell line is known to possess multiple numerical and structural chromosomal abnormalities but to preserve thyroid differentiation qualities (35). We analyzed the HTori-3 cells growing in the lab by partial karyotyping and FISH with the *RET* probe to assure that they contained chromosomes 10 and the *RET* gene loci. Indeed, three intact copies of chromosome 10 were present

in each cell, each containing a single, undisrupted *RET* locus (Fig. 1, *left*). The RT-PCR analysis revealed that these cells expressed *Tg* and *NIS* and, therefore, preserved the most specialized functions of differentiated thyroid cells (Fig. 1, *right*).

Survival of HTori-3 cells was evaluated after radiation doses ranging from 0–50 Gy. The number of surviving cells was calculated by counting detached cells and by trypan blue viability staining. The experiment was carried out in duplicate; the average cell survival was 93.4% after 0 Gy, 91.7% after 0.1 Gy, 90.7% after 1 Gy, 84% after 5 Gy, 22% after 10 Gy, and 0 after 50 Gy. Because 50 Gy radiation resulted in 100% cell death, a range of doses between 0 and 10 Gy was selected for further experiments.

Next, sensitivity of the detection of *RET/PTC1* and *RET/PTC3* rearrangements by RT-PCR and hybridization with specific probes were tested. Nonirradiated HTori-3 cells were mixed with variable numbers of cells carrying *RET/PTC1* and *RET/PTC3* rearrangement. The detection limit was one positive cell in 10^5 negative cells for both types of rearrangement (Fig. 2).

To assure that a cell with the rearrangement would be detected, the 2×10^6 cells in an irradiated flask were divided among 72 culture vessels 4 h postirradiation. Therefore, each well received no more than 3×10^4 cells, and if a well contained only one cell with *RET/PTC*, it would constitute 1 part in 3×10^4, a fraction within the limit of detection.

To determine the optimal time interval between irradiation and cell harvesting, cells were irradiated with 5 Gy and collected after either 0, 2, 5–6, or 9 d, which corresponded to zero, one, three, and five cell divisions, respectively. *RET/PTC* rearrangements were detected only in cells grown for 9 d after irradiation. Based on these data, the time interval of 9 d was selected for the main experiment.

In the main experiment, 16 flasks received no radiation (0 Gy), and four to five replicate flasks were subjected to a single dose of 0.1, 1, 5, and 10 Gy γ-radiation. No *RET/PTC* rearrangement was detected after 0 Gy, indicating an extremely

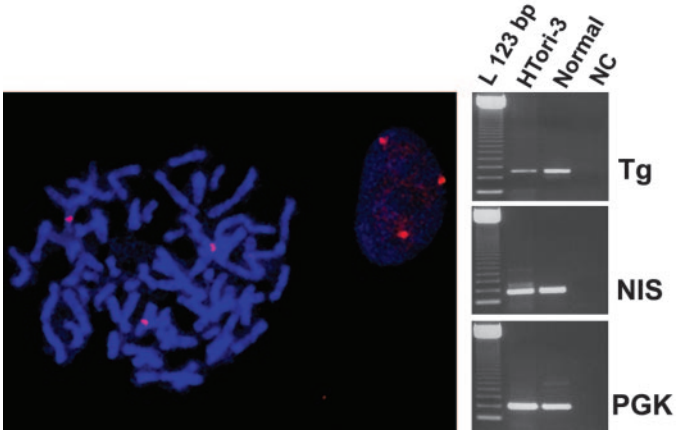

FIG. 1. Characterization of HTori-3 cells used in the study. *Left*, FISH with *RET* probe (*red*) showing a metaphase spread (*left*) and an interphase cell (*right*) with three discrete signals. *Right*, RT-PCR analysis demonstrating the expression of thyroid differentiation genes *Tg* and *NIS* and control gene *PGK* in HTori-3 and normal thyroid cells. L 123 bp, 123-bp ladder; NC, negative control.

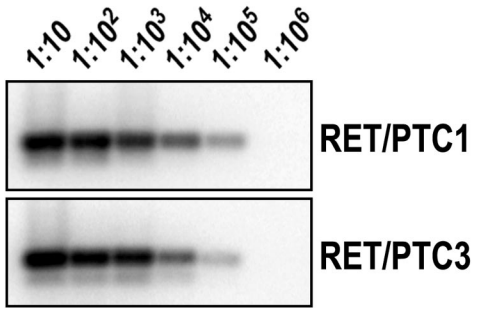

FIG. 2. Detection of *RET/PTC1* and *RET/PTC3* rearrangements in serial dilutions of cell positive for the rearrangement in nonirradiated HT-ori3 cells negative for the rearrangement. The detection limit is $1{:}10^5$ for both types of the rearrangement.

low level of spontaneous generation of *RET/PTC* in this human cell line. All other doses resulted in the generation of *RET/PTC*, with 59 events totally identified (Table 1). Representative RT-PCR blots are shown in Fig. 3. The number of *RET/PTC* events increased with dose over the range 0.1–5 Gy, with the highest number observed after 5 Gy radiation. A dose of 10 Gy resulted in a smaller absolute number of rearrangements detected, although when adjusted for cell survival, the rate of *RET/PTC* per survived cells was comparable with those at 5 Gy (Fig. 4). Statistical analysis for multiple comparisons revealed a significant variation in the mean rate of *RET/PTC* induction after different doses when calculated based on the number of irradiated cells ($P = 0.002$) and surviving cells ($P = 0.02$). Individual comparisons showed that the rate of *RET/PTC* in surviving cells was significantly different between 0 and 0.1 Gy ($P = 0.009$) and 0.1 and 1 Gy ($P = 0.0004$) but not between 1 and 5 Gy ($P = 0.06$). At all doses, *RET/PTC1* was more common than *RET/PTC3*, with the ratio between the two types being at least 2:1.

Discussion

We report here the induction of *RET/PTC* rearrangements in differentiated human thyroid cells after *in vitro* exposure to γ-radiation. *RET/PTC* was detected after 0.1 Gy, the smallest dose applied in this study, and the rate of detection increased proportionally from 0.1–1 Gy. A dose of 5 Gy produced the greatest number of rearrangements per surviving cell, but this number was roughly half that expected from the response at 1 Gy. Importantly, these doses were comparable with those received by the individuals who subsequently developed thyroid cancer in various human populations. Indeed, thyroid doses in the areas surrounding the Chernobyl accident site were in the range of 0.05–4 Gy (8). Thyroid cancer patients previously exposed to therapeutic or environmental radiation received between 0.09–12.5 Gy (40). In addition, in this study, the frequency of *RET/PTC* did not grow further when the dose was increased from 5–10 Gy. Therefore, the rate of *RET/PTC* in surviving cells reached plateau approximately at 5 Gy and then remained constant up to 10 Gy. This, again, parallels the human populations, where thyroid cancer risk associated with radiation exposure has a linear dose response for doses <10 Gy, whereas very high doses pose a low risk (40, 41).

These findings provide additional evidence for a direct

TABLE 1. Number of *RET/PTC* events and rate of *RET/PTC* per irradiated cells and surviving cells

	Dose (Gy)				
	0	0.1	1	5	10
Total number of cells irradiated	33.0×10^6	11.8×10^6	10.6×10^6	11.9×10^6	15.0×10^6
Average survival (%)	93.4	91.7	90.7	84.0	22.0
RET/PTC1	0	2	15	22	8
RET/PTC3	0	0	3	8	1
Total *RET/PTC*	0	2	18	30	9
Average rate of *RET/PTC* per 10^6 irradiated cells (±SD)	0	0.1 ± 0.1	1.6 ± 0.4	3.0 ± 1.4	0.9 ± 0.6
Average rate of *RET/PTC* per 10^6 surviving cells (±SD)	0	0.1 ± 0.1	1.8 ± 0.5	3.6 ± 1.7	4.0 ± 2.9

link between *RET/PTC* rearrangement and radiation exposure. Moreover, the similarity in radiation doses and patterns of dose response, and the fact that the experiment employed human cells that preserved differentiated thyroid cell functions suggest that this *in vitro* model may serve as a valuable tool for studying the mechanisms of *RET/PTC* generation and radiation-induced carcinogenesis in human populations. The limitations of this experimental system include the usage of cultures cells which are immortalized and have significantly higher proliferation rate and the fact that the cells have three copies of the *RET* gene, which is expected to increase the probability of *RET/PTC* rearrangement compared with the diploid human cells.

In this study, *RET/PTC* rearrangement was identified only in cells grown for 9 d after irradiation, which corresponded to approximately five population doublings. There are two possible explanations for this phenomenon: *RET/PTC* was generated in a cell immediately after irradiation, but either chimeric mRNA was not expressed during the initial period or the detection method failed to detect the rearrangement in a single cell, and several cell divisions were necessary to generate a number of cells/mRNA copies sufficient for the detection; or *RET/PTC* was not a result of direct DNA damage and formed later, after several cell divisions. Although the experimental design used in this study could not answer this question, we will attempt to address it in the future.

For all doses used in this study, *RET/PTC1* was significantly more common than *RET/PTC3*. This predominance of *RET/PTC1* is similar to previous observations in normal human thyroid tissues transplanted in SCID mice and exposed to 50-Gy x-rays (34) and recapitulates the findings in tumors from patients subjected to therapeutic external irradiation (30, 31). However, it contradicts the experience with post-Chernobyl thyroid tumors, where *RET/PTC3* was the dominant type of rearrangement in tumors developed less than 10 yr after exposure, whereas those manifested after a longer latency had predominantly *RET/PTC1* (29). The most plausible explanation for this discrepancy is that, despite a smaller number of *RET/PTC3* induced by radiation, this rearrangement confers thyroid cell with higher proliferative activity and tumorigenic potential, so that *RET/PTC3*-positive tumors require less time to reach clinically significant size and, therefore, manifest earlier than those arising through the *RET/PTC1* mechanism. This possibility is supported by some experimental data. Indeed, a difference in the effects of *RET/PTC1* and *RET/PTC3* activation *in vitro* was observed in PC Cl3 rat thyroid cells transfected with both

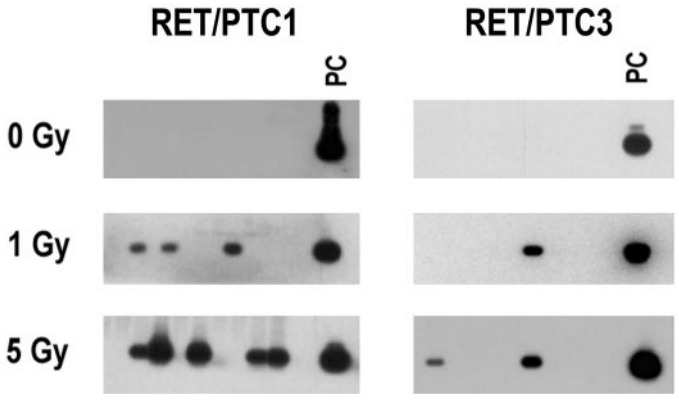

FIG. 3. Detection of *RET/PTC* rearrangements in representative experiments after 0, 1, and 5 Gy irradiation. PC, Positive control.

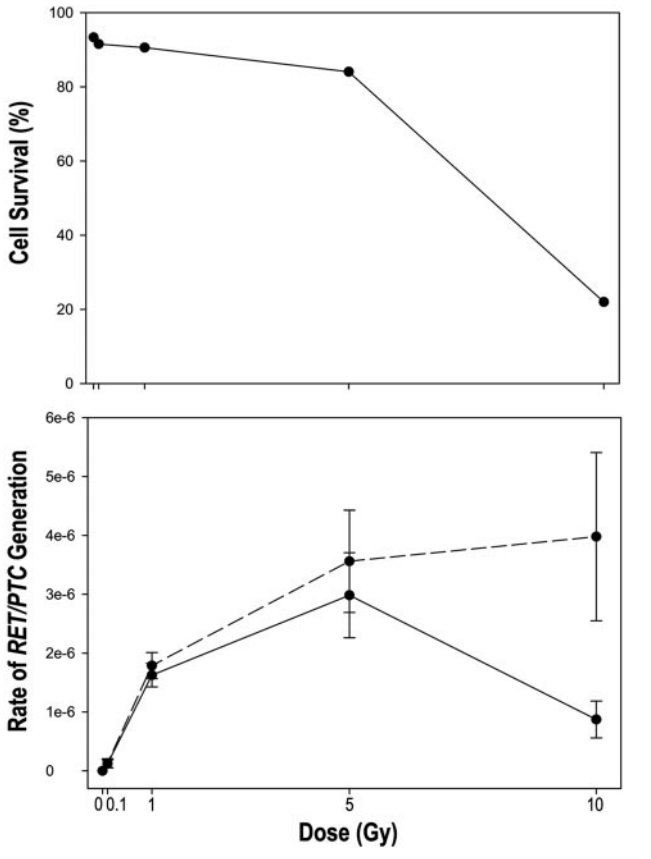

FIG. 4. *Upper panel*, Cell survival; *lower panel*, rate of *RET/PTC* generation per irradiated cells (*solid line*) and surviving cells (*dotted line*) after 0–10 Gy irradiation. Each *point* represents the mean of four to five individual experiments (±SD).

oncogenes (42). The cells expressing *RET/PTC3* had significantly higher proliferation rate and approximately 3-fold higher levels of phosphorylation of the downstream target, mitogen-activated protein kinase. Other evidence comes from studies of transgenic mice. Although thyroid-specific expression of *RET/PTC1* led to the development of slowly progressing and virtually nonmetastatic thyroid cancers (43–45), mice expressing *RET/PTC3* developed aggressive and metastatic thyroid tumors (46). Alternatively, the reverse ratio of *RET/PTC1* and *RET/PTC3* may reflect the difference in radiation sources, *i.e.* the internal exposure to I-131 and other radioiodines after Chernobyl and external γ- or x-ray irradiation in the experiments and in patients exposed to therapeutic radiation. These possibilities can be tested using the *in vitro* model established in this study. In addition, these experiments showed for the first time the induction of *RET/PTC* after doses as low as 0.1 Gy and, therefore, will allow to study the effects of low-dose ionizing radiation. Finally, because this experimental system employs cultured cells that are easy to manipulate on the genetic level, it may help to explore other important issues related to radiation carcinogenesis, particularly the role of specific genes in predisposition to chromosomal rearrangements and, therefore, radiation-induced cancer.

In summary, in this study, we demonstrate a dose-dependent generation of *RET/PTC* rearrangements in human thyroid cells after exposure to 0.1–10 Gy γ-radiation. This provides additional evidence for a direct link between this genetic event and radiation exposure and offers a powerful experimental system for studying radiation-induced carcinogenesis in the thyroid gland.

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