Formation of carcinogenic chromosomal rearrangements in human thyroid cells after induction of double-strand DNA breaks by restriction endonucleases

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Abstract

Ionizing radiation (IR) exposure increases the risk of thyroid cancer and other cancer types. Chromosomal rearrangements, such as *RET/PTC*, are characteristic features of radiation-associated thyroid cancer and can be induced by radiation *in vitro*. IR causes double-strand breaks (DSBs), suggesting that such damage leads to *RET/PTC*, but the rearrangement mechanism has not been established. To study the mechanism, we explored the possibility of inducing *RET/PTC* by electroporation of restriction endonucleases (REs) into HTori-3 human thyroid cells. We used five REs, which induced DSB in a dose-dependent manner similar to that seen with IR. Although all but one RE caused DSB in one or more of the three genes involved in *RET/PTC*, rearrangement was detected only in cells electroporated with either PvuII (25 and 100 U) or StuI (100 and 250 U). The predominant rearrangement type was *RET/PTC3*, which is characteristic of human thyroid cancer arising early after Chernobyl-related radioactive iodine exposure. Both enzymes that produced *RET/PTC* had restriction sites only in one of the two fusion partner genes. Moreover, the two enzymes that produced *RET/PTC* had restriction sites present in clusters, which was not the case for RE that failed to induce *RET/PTC*. In summary, we establish a model of DSB induction by RE and report for the first time the formation of carcinogenic chromosomal rearrangements, predominantly *RET/PTC3*, as a result of DSB produced by RE. Our data also raise a possibility that *RET/PTC* rearrangement can be initiated by a complex DSB that is induced in one of the fusion partner genes.

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Introduction

Ionizing radiation (IR) is a well-known carcinogen linked to a variety of human cancers. One of those is thyroid cancer, which has a well-established link to the exposure to external γ - and X-ray radiation (Schneider 1990, Ron *et al.* 1995, Schlumberger *et al.* 1999) and internal exposure to ingested I-131 (Tuttle & Becker 2000, Cardis *et al.* 2005a). The risk of thyroid cancer has a linear dose response for doses 0.1–2 Gy, with flattening of the dose–response curve at higher doses (Ron *et al.* 1995, Cardis *et al.* 2005a,b).

Over the last decade, strong evidence has been accumulated supporting the central role of chromosomal rearrangements in carcinogenesis initiated by IR (Ciampi *et al.* 2005, Gandhi *et al.* 2010). A prototypic example of such radiation-induced rearrangement is a *RET/PTC* rearrangement, which is highly prevalent in thyroid cancer in individuals exposed to IR (Bounacer *et al.* 1997, Rabes *et al.* 2000, Collins *et al.* 2002) and can be induced in thyroid cells by IR *in vitro* (Ito *et al.* 1993, Mizuno *et al.* 2000, Caudill *et al.* 2005). The two most common rearrangement types are *RET/PTC1* and

RET/PTC3, in which *RET* is fused to either *CCDC6* (*H4*) or the *NCOA4* (*ELE1*) gene (Grieco *et al.* 1990, Santoro *et al.* 1994). *RET/PTC1* is more common after γ -radiation, whereas *RET/PTC3* was the predominant type in patients exposed to I-131 after Chernobyl who developed thyroid cancer <10 years after exposure (Bounacer *et al.* 1997, Nikiforov *et al.* 1997, Rabes *et al.* 2000). *In vitro*, the formation of *RET/PTC* can be found in thyroid cells exposed to external γ - and X-ray radiation (Ito *et al.* 1993, Mizuno *et al.* 2000, Caudill *et al.* 2005). The findings in the human populations and in experimental models provide strong evidence for the direct role of radiation in generating *RET/PTC*. Understanding of the mechanisms of formation of this carcinogenic rearrangement can therefore provide important insights into the mechanisms of radiation carcinogenesis in human thyroid cells and in other cell types.

IR is known to induce various types of DNA damage, of which double-strand breaks (DSBs) are considered to be a crucial primary lesion for a variety of biological end points, including chromosomal aberrations and cell transformation (Bryant & Riches 1989, Winegar *et al.* 1992). However, how exactly radiogenic DSBs lead to chromosomal aberrations remain unclear. Three basic theories have been proposed. The Breakage-and-Reunion theory postulates that chromosomal aberrations arise mainly as a result of rejoining of two DSBs located closely in space and time (two-hit event) (Savage 1998, Hlatky *et al.* 2002). Presumably, most rejoining events occur via nonhomologous end joining (Yates & Morgan 1993, Rothkamm *et al.* 2001). An alternative, one-hit mechanism is suggested by the Molecular theory, which postulates that one radiation-induced DSB is sufficient to initiate an exchange that occurs with an undamaged DNA molecule (Chadwick & Leenhouts 1978, Goodhead *et al.* 1993). The plausible mechanism for such a series of events is homologous recombination initiated by one DSB. The Exchange theory suggests that the initiation lesions are not DNA breaks induced by radiation but rather ‘unstable lesions’ that do not disrupt the continuity of chromosomes but can initiate exchange between two lesions (Revell 1975). Although the Breakage-and-Reunion theory remains most widely accepted, none of the three theories can adequately explain all available experimental data on the dose–effect relationship and complexity of radiation-induced aberrations (Edwards 2002). Moreover, these theories are based on the assumption that primary DNA lesions are directly induced by radiation. However, additional evidence exists that radiation can lead to chromosomal exchanges by an indirect mechanism, i.e. mediated by radiation-induced

genomic instability. This possibility is supported by studies showing the occurrence of new chromosomal aberrations in subsequent generations of a cell exposed to radiation (Huang *et al.* 2003, Little 2003), and by a bystander effect, where aberrations are found in cells plated close to, but not in, the field of irradiation or partial irradiation of a cell cytoplasm (Ludwików *et al.* 2002, Morgan *et al.* 2002, Little *et al.* 2003).

In human tumors, the breakpoints of *RET/PTC* rearrangements typically reside within a 1.8 kb intron 11 of *RET* and in intronic sequences in *CCDC6* (intron 7, 1.7 kb) and *CCDC6* (intron 1, 53.4 kb), allowing for undisturbed splicing and expression of genetic chimeras. Analysis of genomic breakpoints in *RET/PTC3* rearrangements in post-Chernobyl thyroid tumors showed random distribution of the breakpoints within the introns, presence of common regions of microhomology composed of three to five nucleotides at the breaks, and minimal modification of sequences at the breakpoints (Bongarzone *et al.* 1997, Nikiforov *et al.* 1999, Klugbauer *et al.* 2001). We have previously reported a dose-dependent generation of *RET/PTC* rearrangements by γ -radiation in thyroid HTori-3 cells, which are immortalized, partially transformed cells that preserved features of thyroid differentiation (Caudill *et al.* 2005).

IR breaks DNA at random location, which precludes investigation of the role of any particular break in the mechanism of *RET/PTC* formation. By contrast, restriction endonucleases (REs), which can be introduced into mammalian cells by electroporation, cause DSBs at defined chromosomal locations (Bryant 1988, Ager *et al.* 1991, Obe *et al.* 1992). REs have been widely used to study the biological consequences of DSBs in the genome, which include the generation of chromosomal rearrangements, among other effects. A linear dose response has been reported in studies on the frequency of chromosomal aberrations induced by restriction enzymes introduced into mammalian cells (Bryant 1984, Winegar *et al.* 1989, Yates & Morgan 1993).

In this study, we compared the characteristics of DSBs induced by IR and RE in thyroid cells and used RE induction of DSBs to study the generation of *RET/PTC* rearrangements in thyroid cells.

Materials and methods

Cell line and culture conditions

HTori-3 cells, which are normal human thyroid cells transfected with an origin-defective SV40 genome (Lemoine *et al.* 1989), were purchased from the

European Tissue Culture Collection (Salisbury, Wiltshire, UK). They are partially transformed but retain a differentiated phenotype as indicated by the ability to accumulate iodine and produce thyroglobulin (Tg). We have previously shown that our HTori-3 cells express both sodium/iodide symporter and Tg (Caudill *et al.* 2005). Primary cultures of human thyroid epithelial cells were established from surgically removed tissues as described previously (Nikiforova *et al.* 2000).

Cell irradiation

Human thyroid primary cells (2×10^3) or HTori-3 cells were plated in a 35 mm plate with coverslip and 16 h later exposed to a single dose of γ -irradiation from cesium-137 (Gamma Cell 40 irradiator) at a dose rate of 0.58 Gy/min. Cells were exposed to 0.1, 0.5, 1, and 5 Gy γ -irradiation and incubated at 37 °C for different periods, 1, 2, 4, 6, 24, 48, and 72 h, and subjected to γ H2AX (H2AFX) immunofluorescence.

Electroporation of restriction enzymes and detection of *RET/PTC* rearrangements

PvuII, StuI, EcoRV, ScaI, and NruI RE (New England Bio Labs, Ipswich, MA, USA) were electroporated into human thyroid primary cells or HTori-3 cells by a previously described method (Carney & Morgan 1999). Briefly, 2×10^6 cells in 800 μ l PBS were electroporated with different amounts of enzymes at 50 mC charge (400 V and 125 μ F) using Gene Pulser Xcell Electroporator (Bio-Rad). For the detection of *RET/PTC*, in each experiment, the electroporated cells were seeded in 30 T25 flasks (6.6×10^4 cells per flask). To sustain continuous growth, cells were transferred to T75 flasks on day 5 post-electroporation and harvested on day 9. RNA was extracted from each flask using a Trizol reagent (Invitrogen) and mRNA was purified using Oligotex mRNA mini kit (Qiagen). RT-PCR with primers specific for *RET/PTC1* and *RET/PTC3* was performed and *RET/PTC* detection was achieved by Southern blot hybridization of the PCR products with 32 P-labeled oligonucleotide probes specific for each rearrangement type as described previously (Caudill *et al.* 2005). Evidence of *RET/PTC* rearrangement in the cells from a given flask was scored as one *RET/PTC* event.

Electroporation efficacy for all studied enzymes was assayed by γ H2AX immunofluorescence after 4 h post-electroporation. Cell survival after electroporation of each RE was evaluated 24 h post-electroporation by trypan blue exclusion.

γ H2AX immunofluorescence

γ H2AX immunofluorescence was used to confirm DSB formation after irradiation and RE electroporation of human thyroid cells as well as for DSB quantitation. Cells cultured on coverslips were fixed with 4% formaldehyde for 10 min followed by the permeabilization with 0.5% Triton X-100 on ice for 5 min. After washing with PBS, cells were stained with 100 μ l staining solution (20 mM Tris, pH 7.6, 137 mM NaCl, 10% skimmed milk, and 0.5% Tween-20) containing antiphosphorylated histone H2AX primary antibody (Upstate Biotechnology, Buffalo, NY, USA) at 1:1000 dilution for 2 h at 37 °C. Coverslips were washed with PBS twice and incubated with Alexa488-labeled antimouse IgG (Molecular Probes, Eugene, OR, USA) in staining solution (1:200) for 1 h at 37 °C. After washing with PBS, samples were counterstained with DAPI (Suzuki *et al.* 2006). In order to quantify the number of DSBs, γ H2AX foci were counted in individual nucleus scanned using a Leica SP5 TCS 4D confocal laser scanning fluorescence microscope with a 63 $\times$, 1.4 N.A. oil PlanApo objective. Twenty nuclei were scanned for each condition and the digital images were reconstructed using Volocity software (Perkin Elmer, Waltham, MA, USA). Image stacks were subjected to uniform 90% intensity thresholding along with separation of touching objects to demarcate the foci signals. This technique allowed for automatic quantification of γ H2AX foci within the nuclear volume.

Fluorescent *in situ* hybridization

Touch preparations of primary thyrocytes and HTori-3 cells grown in chamber slides were subjected to 3D fixation in 4% paraformaldehyde followed by repeatable freeze–thaw cycles in liquid nitrogen (Cremer *et al.* 2008). *RET*, *NCOA4*, and *CCDC6* probes were labeled by nick translation, hybridized, and analyzed as described previously (Gandhi *et al.* 2006).

Statistical analysis

The two-tailed *t*-test for independent samples was used to compare the number of γ H2AX foci induced by irradiation and RE, distances between specific probe pairs, and rates of *RET/PTC* rearrangement formation in different experiments. The difference was considered statistically significant when *P* was <0.05.

Results

Restriction enzymes simulate IR-induced DSBs in human thyroid cells

First, we evaluated the ability of the RE PvuII to induce DSBs at known chromosomal sites in primary cultures

of cells (PCCs) established from normal human thyroid tissue. DSB formation was monitored by immunostaining for γ H2AX foci, which form at DSB sites (Rogakou et al. 1998, Sedelnikova et al. 2002). We observed that the number of γ H2AX foci generated by PvuII was increasing with the increased dose of enzyme (Fig. 1). The dose response was close to linear when the cells were electroporated in the buffer containing between 5 and 100 U PvuII. Higher doses of PvuII (250 and 500 U) produced very large regions of γ H2AX staining, suggesting that foci had conglomerated. The foci induced by PvuII were diffusely distributed within the nuclear volume, which is similar to what is seen in cells treated with IR. However, in contrast to γ -radiation, which induced foci in 100% of cells, PvuII induced foci in 70–89% of cells. Nevertheless, when only cells that had an increased number of γ H2AX foci were scored, the number of foci induced by 25 and 100 U of electroporated PvuII were

comparable with the number of foci induced by 1 and 3 Gy of IR, respectively, which is the dose range known to produce RET/PTC rearrangements in thyroid cells *in vitro* (Caudill et al. 2005) and induce thyroid cancer in human populations (Ron et al. 1995, Cardis et al. 2005a,b). These data provided evidence that DSB can be induced in human cells by RE in a dose-dependent manner and in quantities and distribution similar to DSB induced by IR doses known to cause carcinogenic chromosomal rearrangements.

Then, we investigated whether the kinetics of induction and repair of DSBs induced by PvuII and by γ -radiation is similar between PCC cells and immortalized HTori-3 thyroid cells. After exposure to γ -radiation, the fraction of cells with γ H2AX foci in both types of cells was highest at the earliest time point examined (15 min post-irradiation) and declined with similar kinetics (Fig. 2A). In both cell types, the fraction of cells with induced foci declined more

Figure 1 Comparison of DSBs induced by γ -radiation and PvuII in primary cultured thyroid cells evaluated based on γ H2AX foci formation. (A) γ H2AX foci distribution in cells. (B) Correlation between the number of induced γ H2AX foci and radiation dose and dose of PvuII.

Figure 2 Kinetics of DSB repair in HTori-3 thyroid cells and primary cell cultures (PCC) induced by different doses of γ -radiation (A) and PvuII (B).

slowly in cells treated with 5 Gy compared with 1 Gy. Similar results were obtained in cells electroporated in the presence of low (25 U) and high (250 U) dose of PvuII, except that it took longer for cells with foci to reach its maximum and return to baseline (Fig. 2B). In both cell types, the fraction of cells with induced γ H2AX foci was higher in cells that were electroporated in the presence of 250 U compared with 25 U PvuII, and this fraction declined more slowly (Fig. 2B). The slower kinetics of focus formation and dissolution after RE treatment were not surprising given the requirement for transport of the enzyme into the nucleus and the known persistence of endonuclease activity for up to 12 h after electroporation (Costa & Bryant 1991), in contrast to the virtually immediate one-time induction of DSBs by IR. These results indicated that both types of cells repaired induced DSBs with similar kinetics. In addition, using 3D fluorescent *in situ* hybridization (FISH) analysis, we determined that HTori-3 cells, known to have three copies of intact chromosome 10, preserve spatial proximity between the *RET*, *NCOA4*, and *CCDC6*

gene loci which has been previously observed in normal human thyroid cells (Nikiforova *et al.* 2000, Gandhi *et al.* 2006; Supplementary Figure 1, see section on supplementary data given at the end of this article). These findings indicate that induction of DSBs by electroporation of RE into a stable cell line (HTori-3 cells) could be used as a model to study the mechanisms of *RET/PTC* rearrangement that arise in human thyroid cells after exposure to IR. Moreover, HTori-3 cells provide a source of large quantity of stably dividing human thyroid cells that are required for such experiments and which cannot be obtained by culturing normal human thyroid cells.

REs induced *RET/PTC* rearrangements with predominance of *RET/PTC3* type

The ability of restriction enzymes to induce *RET/PTC* rearrangements in thyroid cells was tested using HTori-3 cells and five blunt-end cutting REs: PvuII, EcoRV, NruI, ScaI, and StuI. The enzymes chosen induced DSBs in >60% of cells at a dose that allowed more than 70% of the electroporated cells to survive. The panel of enzymes was also chosen in order to test the validity of the three different theoretical models of radiation-induced rearrangement. Consequently, the enzymes used differed with respect to their capacity to cleave the sequences that become joined in *RET/PTC1* and *RET/PTC3* rearrangements. In *RET/PTC1*, intron 11 of *RET* recombines with intron 1 of *CCDC6*. In *RET/PTC3*, intron 11 of *RET* recombines with intron 7 of *NCOA4*. All three pertinent introns have ScaI cleavage sites, but intron 7 of *NCOA4* lacks sites for PvuII and StuI, and intron 1 of *CCDC6* is the only intron that has an EcoRV site (Table 1 and Fig. 3). None of the three introns have an NruI site. Therefore, if two breaks were needed (Breakage-and-Reunion theory), then ScaI would be expected to induce both *RET/PTC1* and *RET/PTC3* because there are sites for

Table 1 Number and characteristics of restriction enzyme digestion sites in genes participating in *RET/PTC* rearrangements

Restriction enzyme	Number of digestion sites and distance between closest restriction sites ^a		
	<i>RET</i> intron 11, 1.8 kb	<i>NCOA4</i> intron 7, 1.7 kb	<i>CCDC6</i> intron 1, 53.4 kb
ScaI	2 (332 bp)	2 (1274 bp)	9 (397 bp)
PvuII	3 (24 bp)	0	22 (327 bp)
StuI	4 (65 bp)	0	10 (551 bp)
EcoRV	0	0	4 (3271 bp)
NruI	0	0	0

^aDistance between closest restriction sites is shown in brackets.

Figure 3 Restriction maps of gene regions that contain breakpoints involved in forming *RET/PTC1* (*RET* intron 11 and *CCDC6* intron 1) and *RET/PTC3* (*RET* intron 11 and *NCOA4* intron 7).

this enzyme in all three of the pertinent genes. Similarly, PvuII and StuI would induce *RET/PTC1* but not *RET/PTC3*, and EcoRV and NruI would not induce *RET/PTC* at all. However, if only one break were needed (the Molecular theory), then only NruI would be expected to fail to induce *RET/PTC*. The Exchange theory, which postulates the formation of initiation lesions that are not DSB, would seem to be not applicable to genomes damaged by restriction enzymes. Nevertheless, if NruI were to induce *RET/PTC*, such a result would support the hypothesis of a mechanism that does not directly involve one or more DSBs in the DNA sequences that recombine.

Induction of *RET/PTC* rearrangements was studied by RT-PCR using the approach we previously used to study the generation of this rearrangement by γ -radiation (Fig. 4; Caudill et al. 2005). *RET/PTC* rearrangements were detected after electroporation with either PvuII (25 and 100 U) or StuI (100 and 250 U) (Table 2). Each enzyme produced both rearrangement types, although *RET/PTC3* was by far the most common type induced by PvuII. The rate of *RET/PTC1* induction by both enzymes was 0.12–0.17 per 10^6 cells, whereas the rate of *RET/PTC3* formation was 0.15–0.34 per 10^6 cells by StuI and 2.19–2.92 by PvuII. The other three restriction enzymes tested did not induce detectable *RET/PTC* rearrangement. Doses of PvuII and StuI that produced *RET/PTC* rearrangements induced between 40 and 200 DSBs per cell, as estimated based on the number of γ H2AX foci.

This amount of DNA damage is in the range induced by 1–5 Gy of IR (Sedelnikova et al. 2002).

Induction of *RET/PTC* rearrangements correlated with clustering of RE cleavage sites in one of the fusion partners

RET/PTC was found in cells treated with either StuI or PvuII, indicating that enzymatic DNA cleavage can cause these types of rearrangements. However, *RET/PTC3* rearrangements greatly predominated in cells electroporated with PvuII, despite the fact that one of the *RET/PTC3* fusion partners, the *PTC3* intron 7, contains no PvuII sites (Table 1 and Fig. 3). The presence of wild-type sequences of *RET* intron 11 and *NCOA4* intron 7 in HTori-3 cells was confirmed by nucleotide sequencing, and PCR products of these intronic sequences showed patterns of digestion expected based on their restriction maps (Supplementary Figure 2, see section on supplementary data given at the end of this article). On the other hand, induction of DSB in one gene was not always sufficient to produce *RET/PTC* because it was not generated by either EcoRV or Scal. These findings indicate that induction of *RET/PTC* was dependent on some other features of DNA damage induced by specific RE. Indeed, a unique feature of PvuII and StuI is that their sites are clustered in *RET* intron 11. Specifically, the intron contains two PvuII sites only 24 bp apart and

Figure 4 Examples of phosphorimages detecting samples positive for *RET/PTC1* (A) and *RET/PTC3* (B) rearrangements induced by 25 U of PvuII restriction endonuclease. PC, positive control.

Table 2 Rate of induction of *RET/PTC* rearrangements by restriction enzymes

Enzyme (units)	<i>RET/PTC1</i>		<i>RET/PTC3</i>	
	Total identified	Rate per 10 ⁶ cells	Total identified	Rate per 10 ⁶ cells
Buffer only	0	0	0	0
PvuII (25)	1	0.12	18	2.19
PvuII (100)	0	0.00	13	2.92
StuI (100)	0	0.00	1	0.15
StuI (250)	1	0.17	2	0.34
Scal (25)	0	0	0	0
Scal (100)	0	0	0	0
EcoRV (25)	0	0	0	0
NruI (100)	0	0	0	0

two *StuI* sites separated by 65 bp (Table 1). By contrast, relevant restriction sites of *EcoRV* and *Scal* are 300 bp or more apart.

Discussion

The results of this study demonstrate that carcinogenic chromosomal rearrangements can be generated in human thyroid cells as a result of DSBs caused by restriction enzymes. The predominant type of the fusion was *RET/PTC3*, which is characteristic of human thyroid cancer arising after exposure to I-131. Moreover, our results raise a possibility that *RET/PTC* can be formed in human thyroid cells as a result of a complex DSB produced in one of the genes participating in the fusion.

The preferential formation of *RET/PTC3* after electroporation of RE found in this study was unexpected because *RET/PTC1* is a predominant type of the rearrangement in most human populations and in experimental exposures to IR (Bounacer *et al.* 1997, Collins *et al.* 2002, Hamatani *et al.* 2008). The notable exception is a population of individuals exposed predominantly to radioiodines as a result of the Chernobyl accident who developed thyroid cancer during the first 10 years after exposure, where *RET/PTC3* was much more common than *RET/PTC1* (Nikiforov *et al.* 1997, Smida *et al.* 1999, Thomas *et al.* 1999, Rabes *et al.* 2000). The reason for *RET/PTC3* prevalence in this population was not clear, and this was believed to represent a sporadic phenomenon characteristic of this population only. However, the results of this study indicate that the same type of human thyroid cells (HTori-3) form predominantly *RET/PTC3* after induction of DSBs by RE, and *RET/PTC1* after exposure to γ -radiation (Caudill *et al.* 2005). This suggests that the choice of rearrangement

type is not random and not cell-type specific but rather is a manifestation of the underlying mechanisms, which can be further studied using a model established in this study.

One possible explanation for the difference between the predominance of *RET/PTC3* over *RET/PTC1* in human populations and after RE electroporation compared with γ -radiation may be related to the mode of DSB generation. Both I-131 (half-life of 8.04 days) and *PvuII* endonuclease activities (known to persist for at least 12 h after electroporation (Costa & Bryant 1991)) result in continuous induction of DSBs, in contrast to a one-time action of γ -radiation.

RET/PTC events in this study were detected after inducing DNA damage with two out of five REs used. It is unlikely that the failure of some REs to induce *RET/PTC* reflects failure to cause DSB because all the enzymes generated a similar number of γ H2AX foci. It is possible that the ability to induce *RET/PTC* depends on the location and characteristics of the induced breaks. *RET* intron 11 contains a pair of *PvuII* sites and a pair of *StuI* sites, the two enzymes that induced *RET/PTC*. Cleaving at both sites would cause a small deletion and a DSB. Perhaps such damage is less prone to repair and is therefore more likely to elicit a different repair outcome, such as *RET/PTC*. Incidentally, *PvuII* has two restriction sites located on a shorter distance than *StuI*, and it was approximately tenfold stronger as an inducer of *RET/PTC3*. It is interesting to note that both γ -radiation and I-131 are known to produce clustered breaks and breaks that cannot be ligated because they lack the required 5'-phosphate and/or 3'-hydroxyl group (Goodhead 1994, Sutherland *et al.* 2000, 2002). The I-131 isotope, which emits both γ -radiation and β -particles, has an even higher potential for producing complex DNA damage. The charged particles have more complex track structure and denser energy deposition than γ -radiation, with higher chance of inducing DSBs and clustered DNA damage (Goodhead 1994, Hada & Georgakilas 2008). In thyroid cells, I-131 induces DSBs detectable as γ H2AX or 53BP1 foci (Hershman *et al.* 2011), as well as clustered DNA damage detected by Comet assay (Grzesiuk *et al.* 2006). Taken together, these data and results of our study raise at least a theoretical possibility that complex DSB in one gene is sufficient to initiate the generation of *RET/PTC* rearrangement in thyroid cells. This would support a one-hit mechanism postulated by the Molecular theory (Chadwick & Leenhouts 1978, Goodhead *et al.* 1993).

Alternately, it remains possible that the formation of *RET/PTC3* occurred via two breaks, the first being formed by *PvuII* cutting at its canonical recognition

sites and the second being formed in the *NCOA4* gene by nonspecific cleavage. *In vitro*, under artificial conditions, PvuII can cleave DNA at sites that match only five of the six bases in the canonical PvuII cleavage site (Nasri & Thomas 1987, Robinson & Sligar 1995). However, such star activity has not been observed *in vivo* (Nastri et al. 1997, Simoncsits et al. 2001), and inspection of *RET/PTC3* junctions found no evidence of breaks made by PvuII star activity. Furthermore, nonspecific cutting has not been shown for StuI. Therefore, the data do not support nonspecific cleavage by any known capability of PvuII.

In summary, in this study, we established a model of DSBs induced by various REs in human thyroid cells and report for the first time the formation of a carcinogenic chromosomal rearrangement after RE electroporation. The results obtained using this model suggest that complex DSB produced in one of the fusion partners may be sufficient for the formation of *RET/PTC* in thyroid cells. Moreover, this model can be used for further studies of radiation carcinogenesis in human thyroid cells, particularly in light of a similarity of *RET/PTC* types induced by RE and by radioiodine.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-11-0314>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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