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MECHANISMS OF CHROMOSOMAL REARRANGEMENTS IN SOLID TUMORS: THE MODEL OF PAPILLARY THYROID CARCINOMA

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Abstract

Thyroid cancer, and its most common type, papillary carcinoma, frequently have chromosomal rearrangements and therefore represents a good model for the understanding of mechanisms of chromosomal rearrangements in solid tumors. Several types of rearrangement known to occur in thyroid cancer, including *RET/PTC*, *NTRK1* and *BRAF/AKAP9*, are more common in radiation-associated thyroid tumors and *RET/PTC* can be induced experimentally by exposing human thyroid cells to ionizing radiation. In this review, the molecular mechanisms of generation of *RET/PTC* and other chromosomal rearrangements are discussed, with the emphasis on the role of nuclear architecture and interphase gene proximity in the generation of intrachromosomal rearrangements in thyroid cells.

Thyroid cancer and chromosomal rearrangements

Thyroid cancer is the most common malignant tumor of the endocrine system and accounts for approximately 1% of all newly diagnosed cancer cases [1]. Papillary thyroid carcinoma is the most prevalent type of thyroid malignancy and constitutes ~80% of all thyroid cancers. More than 70% of papillary carcinomas have known genetic alterations all of which lead to the activation of the mitogen-activated protein kinase (MAPK) signaling pathway [2–4]. These abnormalities include chromosomal rearrangements (intrachromosomal inversions and interchromosomal translocations) and point mutations. Most common point mutations involve the *BRAF* gene as well as *RAS* genes [5,6]. The most common chromosomal rearrangement involves the *RET* gene and is called *RET/PTC* [7,8]. In addition to *RET/PTC*, chromosomal rearrangements involving the *NTRK1* and *BRAF* genes also occur in papillary thyroid carcinomas, although with a significantly lower prevalence [9,10]. As a result, papillary thyroid carcinoma represents a good model to study the mechanisms of chromosomal rearrangements in solid tumors.

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Radiation-induced thyroid cancer involves chromosomal rearrangements rather than point mutations

Exposure to ionizing radiation is a well known risk factor for thyroid cancer, especially for papillary carcinoma. An increased incidence of thyroid cancer has been documented after therapeutic use of ionizing radiation during childhood [11,12] as well as after accidental environmental exposure. The latter includes survivors of the atomic bomb explosion in Hiroshima and Nagasaki in 1945 [13,14] and of the nuclear test fallout in the Marshall Islands in 1954 [15,16] and those exposed to radiation after the Chernobyl nuclear disaster in 1986 [17–19]. Studied of thyroid cancer in various populations revealed a sharply different prevalence of chromosomal rearrangements and point mutations in tumors from individuals exposed to ionizing radiation as compared to sporadic tumors, i.e. arising in patients with no radiation history [10,20] (Table 1). Indeed, the prevalence of *RET/PTC* is very high in individuals with a history of radiation exposure. This includes those subjected to either accidental (mostly radioiodine) irradiation or therapeutic (mostly external beam) irradiation, as 50–80% of those papillary carcinomas harbor *RET/PTC* [21–23]. In contrast, in the general population clonal *RET/PTC* rearrangements are seen in 10–40% of papillary carcinomas in most studies, although the reported prevalence varies dramatically [24,25], largely due to different sensitivities of the techniques used for their detection [26,27]. Higher prevalence of *RET/PTC* is seen in pediatric tumors [23,28,29], a significant portion of which may be associated with radiation exposure. Another chromosomal rearrangement, *BRAF/AKAP9* is also found predominantly in papillary carcinomas associated with radiation exposure [10]. The opposite is true for point mutations, such as those involving the *BRAF* gene. *BRAF* V600E point mutation represents the most common genetic alteration in sporadic papillary carcinomas, being found in 40–45% of those tumors [30,31], but it is rarely found in radiation-related tumors [32]. Moreover, among papillary carcinomas in atomic bomb survivors in Japan, the presence of *RET/PTC* directly correlated with the dose of radiation, whereas the inverse correlation was found between the dose and *BRAF* point mutations [33,34]. These findings provide evidence that generation of chromosomal rearrangements in human thyroid carcinomas is closely linked to radiation exposure.

Types of *RET/PTC* rearrangement in sporadic and radiation-induced cancers

RET/PTC rearrangement is formed by fusion between the 3’ portion of the *RET* gene, coding for the receptor tyrosine kinase, and the 5’ portion of various unrelated genes. The two most common rearrangement types, *RET/PTC1* and *RET/PTC3*, are paracentric inversions since both *RET* and its respective fusion partner, *H4* or *NCOA4 (ELE1; RFG, ARA70)*, reside on the long arm of chromosome 10 [8,35,36] *RET/PTC2* and nine more recently identified types of *RET/PTC* are all interchromosomal translocations [37,38].

In most populations, *RET/PTC1* is the most common type of *RET/PTC* as it comprises 60–70% of positive cases, whereas *RET/PTC3* accounts for 20–30%, and *RET/PTC2* and other novel rearrangement types for less than 5–10% [24,25]. In individuals exposed to accidental or therapeutic radiation, *RET/PTC1* remained to be the most common rearrangement type except for the tumors that developed less than 10 years after radiation exposure at Chernobyl, where *RET/PTC3* was the predominant rearrangement type [21,22,39,40].

Experimental evidence for the association between *RET/PTC* rearrangements and radiation exposure

The association between *RET/PTC* rearrangement and ionizing radiation is supported by several studies demonstrating the induction of *RET/PTC* by irradiation of cultured human thyroid cells [41,42] and of human fetal thyroid tissue xenografts in SCID mice [43,44]. It has

been shown that exposure of HTori-3 human thyroid cells to physiologically relevant doses of gamma-radiation (0.1–10 Gy) resulted in a dose-dependent generation of both *RET*/*PTC1* and *RET/PTC3* rearrangements [42]. In this study, *RET/PTC1* was more common than *RET/PTC3* after each dose, comprising 80% of all rearrangements.

Although the dose of exposure were significantly higher (50–100 Gy) in two studies that employed human fetal thyroid tissue xenografts, they demonstrated that X-ray irradiation led to the generation of both *RET/PTC*1 and *RET/PTC*3 rearrangements, with *RET/PTC1* type being the most common [43,44]. These studies provide evidence for the direct link between exposure to ionizing radiation and generation of *RET/PTC* rearrangement in human thyroid cells.

Molecular mechanisms of chromosomal aberrations induced by radiation

Ionizing radiation damages DNA in a variety of ways as a result of either direct energy deposition along the radiation track or by secondary reactive oxygen species produced by ionization of water. It is known that 1 Gy of X-ray radiation produces 500–1000 single-strand DNA breaks, 20–40 double-strand breaks (DSBs), >3000 damaged bases, and ~150 DNA-protein crosslinks per cell [45,46]. Of these types of DNA damage, DSBs are considered to be a crucial primary lesion for a variety of biological end points, including cell killing, chromosomal aberrations, and cell transformation [47,48]. However, how exactly radiogenic DSBs lead to chromosomal rearrangements remains not fully understood. Several basic theories have been proposed [49–51]. The most widely accepted is the Breakage-and-Reunion theory. It postulates that chromosomal aberrations arise mainly as a result of rejoining of two DSBs located closely in space and time (two-hit event) [49,50]. Presumably, most rejoining events occur via non-homologous end joining (NHEJ) [52,53]. The initial distribution of primary breaks is assumed to be random, although the rejoining efficiency is expected to be influenced by their proximity. 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 [54,55]. The only 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 [56].

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 [57]. Moreover, these theories are based on the assumption that primary DNA lesions, either DSBs or less well-defined “unstable lesions,” are directly induced by radiation (direct mechanism). However, there is at least a theoretical possibility that radiation can lead to chromosomal exchanges entirely by the indirect mechanism, i.e. mediated by radiation-induced genomic instability and not involving the actual breaks induced by radiation. This possibility is supported by studies showing the occurrence of new chromosomal aberrations in subsequent generations of a cell exposed to radiation [58,59], 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 [60–62].

Interphase gene proximity provides structural basis for the generation of *RET/PTC* rearrangement

It appears that nuclear architecture contributes to the generation of *RET/PTC* and other recurrent chromosomal rearrangements found in cancer cells by placing potentially recombinogenic chromosomal loci in close proximity in the interphase nuclei of human cells

(Fig 1). For *RET/PTC*, this was initially demonstrated for the *RET* and *H4* genes in a study that utilized fluorescence in situ hybridization (FISH) and three-dimensional (3D) confocal microscopy and showed that these genes were non-randomly located with respect to each other in the interphase nuclei of human thyroid cells and were much closer than expected based on their genomic separation [63]. In fact, at least one pair of *RET* and *H4* were found juxtaposed in more than one third of adult thyroid cells. This study also showed that the proximity between potentially recombinogenic genes was cell-type specific and was not present in some non-thyroid cells such as mammary epithelial cells. More recently, similar finding were provided for *RET* and *NCOA4*, the partners of *RET/PTC3* rearrangement [64]. Using FISH and high-resolution 3D confocal microscopy, it was shown that *NCOA4* was located closer to *RET* than expected based on their genomic separation. In addition, spatial proximity was found to exist between the partners of another rearrangement occurring in papillary thyroid cancer, *TRK* [65]. Utilizing both 2D distance measurements and 3D mathematical projection, *NTRK1* was shown to be closer to its translocation partner, *TPR*, in thyroid cells but not in lymphocytes.

It is likely that spatial proximity represent a pre-requisite for most rearrangements in human tumors, including intrachromosomal and interchromosomal exchanges. Thus, *BCR* and *ABL* genes, which are located on different chromosomes and frequently rearranged in leukemias, were located close to each other in normal human lymphocytes [66]. Likewise, *MYC*, *BCL* and immunoglobulin loci, which are located on different chromosomes and recombined in various types of B-cell lymphoma, were shown to be preferentially positioned in close spatial proximity relative to each other in normal B cells [67].

Irrespective of the specific DNA repair mechanism involved in recombination, spatial proximity is likely to predispose to specific rearrangements by making the neighboring regions prone to simultaneous damage by radiation or other DNA-damaging agents, and/or by facilitating mis-rejoining of free DNA ends located immediately adjacent to each other. Since the nuclear architecture is cell type specific, it may also provide an explanation why, in contrast to point mutations, almost all cancer-related chromosomal rearrangements are specific for particular cell/tumor types.

It remains unclear why specific chromosomal regions are located close to each other. For genetic loci located on the same chromosome, this is likely to involve high order chromosome folding that would allow the genes to be positioned non-randomly with respect to each other. It is known that double stranded DNA is wrapped around histones forming nucleosomes which are then arranged in a 30 nm fiber, solenoid structure [68]. Diverse models varying from irregularly folded chromatin fibers [69], radial loops [70,71], giant loops [72] to the random walk/giant loop model [73] have been proposed for higher order interphase chromatin compaction with the eventual packaging of interphase chromosomes into well defined chromosomal territories (CTs) [74]. With respect to the 18 Mb region on 10q containing *RET*, *NCOA4*, and *H4*, evidence for the large-scale helical folding of this chromosomal region in the interphase nuclei of human thyroid cells was provided [64]. This pattern of chromatin folding can offer the basis for proximity between *RET* and *NCOA4* and *H4*. Whether or not such folding represents a unique structure of this chromosomal region or is a universal feature of interphase chromosome organization remains unknown.

Location of genes within chromosomal territories may influence the type of recombination

A peculiar feature of rearrangements found in papillary thyroid cancers is that almost all of them are intrachromosomal inversions rather then interchromosomal translocations. Indeed, in addition to *RET/PTC1* and *RET/PTC3* that involve genes on 10q11.2–q21, the *TRK* rearrangements most commonly involve the *NTRK1* (1q21–q22) fusion to either *TPR* (1q25)

or *TPM3* (1q25) [9] and recently indentified *BRAF/AKAP9* rearrangement involve two genes located on 7q [10]. A recent study provides experimental evidence suggest that the predominance of intrachromosomal recombination in thyroid cells may also be in part due to the nuclear architecture [75]. In this study, the location of specific chromosomal loci involved in intrachromosomal and interchromosomal exchanges in thyroid cells were analyzed. Simultaneous hybridization with gene-specific probes and their respective whole chromosome paints was used to establish the positioning of specific recombinogenic loci within their chromosome territories (CTs). It was found that genes involved in intrachromosomal rearrangements were positioned at significantly greater distances away from the CT edge and internally within their CTs as compared to genes involved in translocations that were positioned closer to the CT edge [75]. The frequent location of *RET* and its recombinogenic partners within the interior of the chromosomal territory, surrounded by its own chromosomal material and with limited availability to interact with neighboring chromosomal territories, is likely to predispose it to intrachromosomal exchange, such as seen in most cases of *RET/PTC* (Fig 2). Similar findings have been obtained in another study that demonstrated a significant correlation between the extent of intermingling between different CTs and frequency of translocation involving specific chromosome pairs [76].

Potential DNA repair mechanisms involved in *RET/PTC* rearrangement

In mammalian cells, DSBs are repaired by two general pathways that are based on homology-dependent or nonhomologous recombination. The homology-dependant mechanism encompasses several pathways such as homologous recombination repair (HRR), single strand annealing (SSA), and non-allelic homologous recombination (NAHR). Nonhomologous mechanism is known as nonhomologous end joining (NHEJ). Another recently described repair pathway, microhomology mediated end joining (MMEJ), combines features of the two major pathways as it joins DNA ends after preliminary aligning them using short homology DNA sequences located distant to the break. These repair pathways utilize common enzymatic factors as well as those distinct to specific repair mechanisms. Usage of ATM/ATR and NBS1 kinases as the primary DSB sensors is common for homology based and non-homologous repair [77]. However, DNA ends are hold together and initially processed by different enzymes, DNA-PKs (Ku70/Ku80) in NHEJ [78] and Rad52 in HHR and SSA [79]. In all pathways, the processing of DNA ends and trimming is carried by conserved multiprotein MRE11/Rad50/NBS1 (MRN) complex, which plays an important role in DSB repair, meiotic recombination and telomere maintenance [80,81]. SSA and MMEJ additionally require the use of ERCC1-XPF (Rad10-Rad1) complex to incise double-stranded DNA at the junction with single-stranded DNA, nicking bubble structures and 3’ single-strand overhangs [82]. After homology search, strand annealing and end processing DNA integrity is restored. NHEJ employs XPCC4 and Lig4 to ligate the DNA ends [78,83,84].

Several mechanisms have been proposed for the formation of *RET/PTC* rearrangement. They include HRR, NHEJ, SSA, and MMEJ [85–87]. While the genomic sequence of *RET/PTC1* fusion point is difficult to obtain due to a very large size of intron 1, which is a breakpoint cluster region of the *H4* gene, the genomic sequences of 31 *RET/PTC3* fusions from post-Chernobyl thyroid tumors have been reported [85–87] and can be used for the analysis (Table 2).

NHEJ utilizes microhomology (2–4 nt) at DNA ends, and frequently produces microdeletions/insertions at the breakpoints, usually joining the corresponding ends by fast end processing [88]. The nucleotide sequence feature of NHEJ is the presence of microhomology regions located immediately at the fusion points. In addition, sequence modifications, including small deletions and insertions, are common at the fusion point. Among 31 post-Chernobyl tumors with reported *RET/PTC3* genomic sequence [85–87], 55% of cases had 3–5 nucleotide

homology located at the break (Fig. 3A). Modifications at breakpoints, typically small deletions, were present in 26 (84%) of post-Chernobyl tumors with *RET/PTC3*. In addition to microhomology located immediately at breakpoints, NHEJ may utilize short homology regions located up to 60–300 nt away from the break, as it has been shown in prokaryotic cells [89, 90]. In post-Chernobyl tumors with *RET/PTC3*, microhomology regions located within 50 nt from breakpoint were seen perfectly aligned relatively to the breakpoint in 58% of cases and with 1–2 nucleotide shift in 68% (Fig. 3B). Overall, microhomology regions were present at the breaks or on adjacent to the breaks in 97% of *RET/PTC3* fusions, making the NHEJ pathway a strong candidate in the formation of *RET/PTC* products.

MMEJ is another repair pathway that utilizes short homology sequences. It has been reported that nuclear extracts from urothelial cancers repair DSBs preferentially by MMEJ compared to normal urothelial cell extracts [91]. Characteristic attributes of MMEJ are the utilization of 5–25 nt homology stretches and the presence of deletions flanking the breaks [92]. It has been suggested that high levels of DNA damage can induce MMEJ over typically predominant NHEJ [93]. Among *RET/PTC* fusions, 19% (6 out of 31) had 5 or more nucleotides in homologous regions, and another 49% (15 from 31) had 5–10 nucleotides imperfect homology regions with inserted base(s) between short homologous sequences (Fig. 3C). Overall, 61% of fusions had 5 nt homology stretches and deletions at the fusion point, suggesting that MMEJ may also serve as a mechanism for *RET/PTC* rearrangement in many cases.

SSA and NAHR utilize the repeatable DNA elements for alignment of broken DNA strand(s) and in quiescent cells have typically a limited participation in DSB repair. However, the loss of NHEJ due to down-regulation of its key factors leads to higher incidence of SSA and NAHR [79,90]. Of these two repair pathways, NAHR is unlikely to play a significant role in the generation of *RET/PTC* rearrangements because of the requirement for non-canonical DNA structures (Z-DNA in CG reach DNA regions) at the site of recombination, which are not present in the *RET/PTC* breakpoint cluster regions [94]. SSA utilizes homology regions larger than 15 nt and induces recombination between direct repeats with concomitant loss of one or more repeat units [95]. In model systems, tandem direct repeats serve the best for SSA, but in living cell SSA may use not only direct tandem repeats but also mirror and inverted repeats and repeats dispersed throughout flanking regions of the breaks [85,95]. The available *RET/PTC3* fusion sequences revealed no 15 nt stretches of tandem repeat homology in any of the cases. However, dispersed homologous di-, tri- or tetranucleotide repeats in both fusion partners could be found in 22 (71%) cases. In addition, 16 of those 22 sequences had a deletion involving at least one repeat copy (Fig. 3D). Thus, SSA may be an additional potential repair mechanism for *RET/PTC* rearrangement.

These data, which are based on the analysis of DNA sequences at the fusion points, suggest that the generation of *RET/PTC* rearrangement may involve several possible DNA repair mechanisms, particularly NHEJ and MMEJ, and to lesser extent SSA. It remains unknown whether all of these mechanisms contribute to the generation of *RET/PTC* with similar frequency and if the choice is determined by specific conditions and/or individual genetic background.

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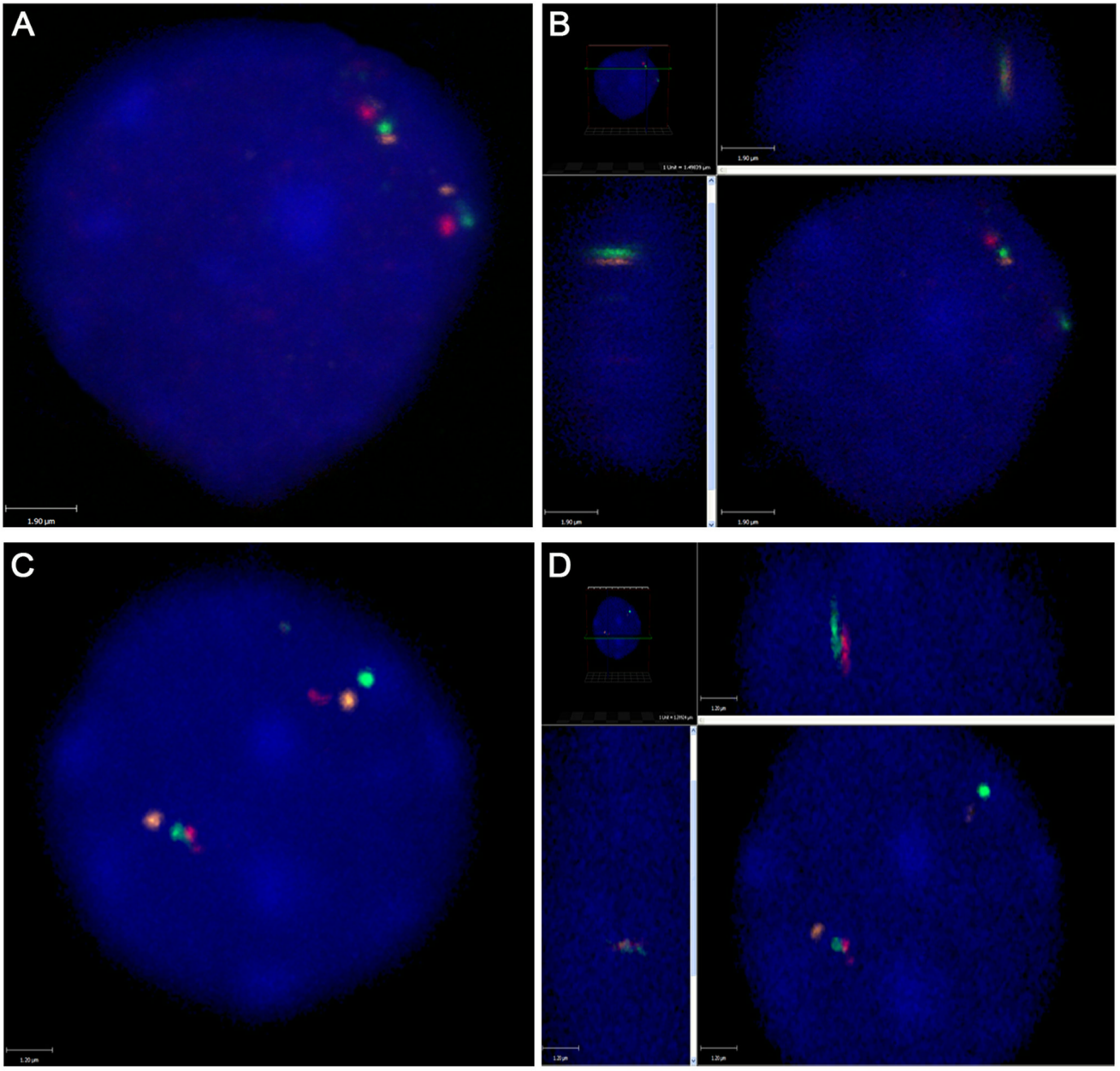

Figure 1.
Three-color fluorescence in situ hybridization (FISH) showing positioning of *RET* (green), *NCOA4* (orange) and *H4* (red) in interphase nuclei of thyroid cells. **A.** 2D image of a nucleus showing two sets of *RET*, *NCOA4* and *H4* with one pair of *RET* and *NCOA4* positioned close to each other. **B.** 3D image showing that *RET* and *NCOA4* are juxtaposed to each other in the same z plane. **C.** 2D image of a nucleus showing one pair of *RET* and *H4* positioned close to each other. **D.** 3D image showing that *RET* and *H4* are juxtaposed to each other in the same z plane.

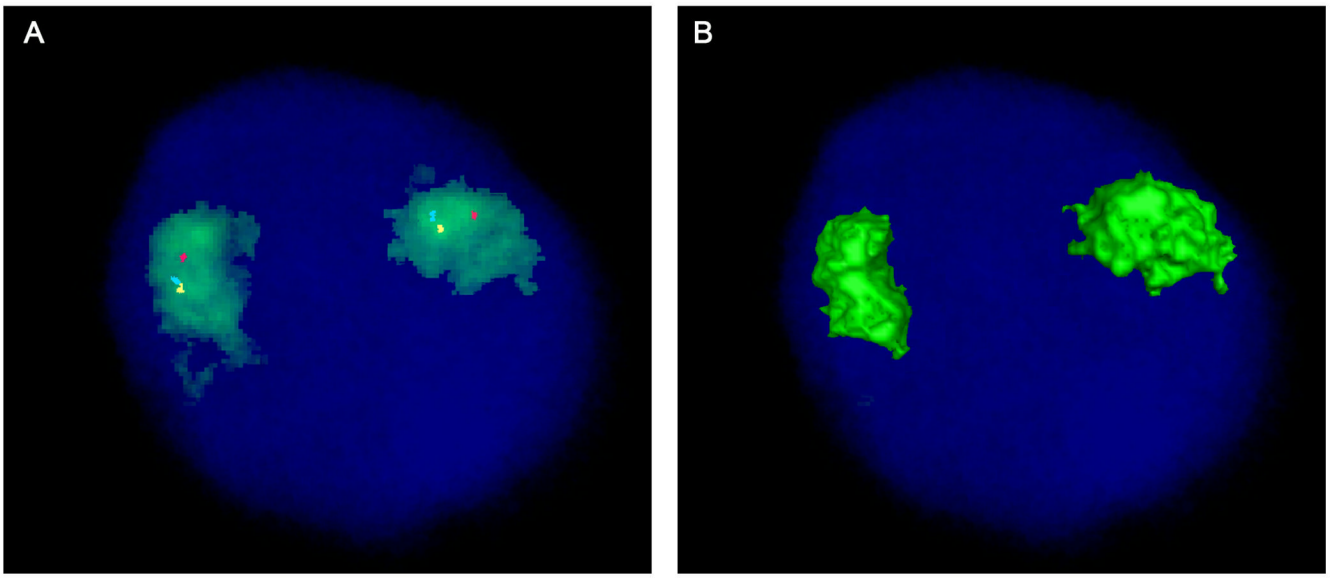

Figure 2.
Four-color FISH showing chromosome 10 territory (green) and location of *RET* (blue pseudocolor), *NCOA4* (yellow pseudocolor) and *H4* (red). **A.** All three genes, *RET, NCOA4* and *H4,* are positioned within the chromosome 10 territory and away from the CT edge. **B.** 3D rendered image showing no signals on the surface of the CT due to the gene positioning inside the CT.

A.

Tumor C24 (microhomology at the break)

RET
5'- GCATCCGGAGCAGTCCCAAGTG**GGC**//
5'-CGGAAGGCTGAGGCAGGAGAAT**GGC**//
ELE 1

Tumor M12T (microhomology at the break and 4nt deletion in *ELE1*)

RET
5'-CTGT**CTG**/ /**CCT**TCTG-3'
5'-GTAC**CTG**/CTTC/**CCT**GAAA-3'
ELE1

B.

Tumor M219T (perfect 6 nt homology)

RET
//TCTCCAAAGCAG**TCATAA**TTGTTCT-3'
//CACCAGAGTCCT**TCATAA**ACCCAGT -3'
ELE1

Tumor C2 (imperfect homology)

RET
//ATTCTGCTTACGC**TT**T**AAG** **ACT**TTG-3'
//CCCTGGTCAGAG**TT**C**AAG**T**ACT**GGG-3'
ELE 1

C.

Tumor C27 (dispersed CCT repeats and 15nt deletion in *RET* containing 2 CCT repeat units)

RET
CCTCT**CCT**GGTGGTGG**CCT**GCC**C** /**CT**TCAGTGTT**CCT**AC/ TAGCA
CCGT**CCT**GCGGGTTCATGCCATTCT//**CCT**G**CCT**CAG**CCTCCT**GA
ELE1

Figure 3.
Representative examples of sequences at *RET/PTC3* breakpoints (//) with DNA-based characteristics for NHEJ (A), MMEJ (B) and SSA (C).

Table 1

Prevalence of chromosomal rearrangements and point mutation in sporadic and radiation-induced papillary thyroid carcinomas

Genetic alteration [ref]	Sporadic Tumors (%)	Radiation-Induced Tumors (%)
RET/PTC rearrangement [21–24,26,39]	10–40	50–85
TRK rearrangement [21,96]	< 5	6
BRAF rearrangement [10]	1	11
BRAF point mutation [30–32,97–99]	40–45	0–4
RAS point mutation [100–102]	10–15	0

Table 2

DNA sequence features of *RET/PTC3* breakpoints in post-Chernobyl tumors and their correspondence to specific DNA repair pathways

	NHEJ					
			MMEJ		SSA	
case ID*	**microhomology at breakpoint**	**distant microhomology (less than 5 nt)**	**distant microhomology (5 or more nt)**	**Deletions at breakpoint**	**repeats in both genes**	**deleted repeats**
C2[2]	**yes**	**yes**	**yes**	**yes**	**yes**	**2 copies**
C8[2]	no	**yes**	no	yes	yes	none
C10[2]	**yes**	**yes**	**yes**	**yes**	yes	none
C11[2]	no	**yes**	yes	no	yes	none
C14[2]	**yes**	**yes**	**yes**	**yes**	yes	none
C15[2]	**yes**	**yes**	yes	no	none	none
C17[2]	no	**yes**	yes	no	none	none
C20[2]	no	**yes**	no	yes	none	none
C24[2]	**yes**	**yes**	no	yes	none	none
C27[2]	**yes**	**yes**	no	yes	**yes**	**2 copies**
C28[2]	**yes**	**yes**	**yes**	**yes**	none	none
C30[2]	no	**yes**	no	yes	none	none
M2T[1]	**yes**	**yes**	no	yes	**yes**	**1 copy**
M12T[1]	**yes**	**yes**	no	yes	**yes**	**1 copy**
M80T[1]	**yes**	**yes**	**yes**	**yes**	**yes**	**2 copies**
M81T[1]	no	**yes**	**yes**	**yes**	**yes**	**3 copies**
M89T[1]	no	no	no	yes	**yes**	**3 copies**
M122T[1]	**yes**	**yes**	**yes**	**yes**	**yes**	**1 copy**
M129T[1]	no	**yes**	**yes**	**yes**	yes	none
M153T[1]	**yes**	**yes**	**yes**	**yes**	**yes**	**1 copy**

	NHEJ					
			MMEJ		SSA	
case ID*	**microhomology at breakpoint**	**distant microhomology (less than 5 nt)**	**distant microhomology (5 or more nt)**	**Deletions at breakpoint**	**repeats in both genes**	**deleted repeats**
M161T[1]	**yes**	**yes**	**yes**	**yes**	**yes**	**2 copies**
M162T[1]	**yes**	**yes**	**yes**	**yes**	**yes**	**1 copy**
M190T[1]	**yes**	**yes**	**yes**	**yes**	none	none
M216T[1]	no	**yes**	**yes**	**yes**	**yes**	**1 copy**
M219T[1]	no	**yes**	**yes**	**yes**	none	none
M225T[1]	**yes**	**yes**	**yes**	**yes**	**yes**	**1 copy**
M259T[1]	**yes**	**yes**	**yes**	**yes**	**yes**	**2 copies**
M263T[1]	no	**yes**	**yes**	**yes**	**yes**	**1 copy**
CH4[3]	no	**yes**	**yes**	**yes**	**yes**	**1 copy**
CH8[3]	no	**yes**	**yes**	**yes**	none	none
CH10[3]	no	**yes**	yes	no	yes	none

**RET/PTC3* sequences reported by

[1] Klugbauer et al. [85]

[2] Nikiforov et al. [86]

[3] Bongarzone et al. [87].