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ORIGINAL ARTICLE

DNA breaks at fragile sites generate oncogenic *RET/PTC* rearrangements in human thyroid cells

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Human chromosomal fragile sites are regions of the genome that are prone to DNA breakage, and are classified as common or rare, depending on their frequency in the population. Common fragile sites frequently coincide with the location of genes involved in carcinogenic chromosomal translocations, suggesting their role in cancer formation. However, there has been no direct evidence linking breakage at fragile sites to the formation of a cancer-specific translocation. Here, we studied the involvement of fragile sites in the formation of *RET/PTC* rearrangements, which are frequently found in papillary thyroid carcinoma (PTC). These rearrangements are commonly associated with radiation exposure; however, most of the tumors found in adults are not linked to radiation. In this study, we provide structural and biochemical evidence that the *RET*, *CCDC6* and *NCOA4* genes participating in two major types of *RET/PTC* rearrangements, are located in common fragile sites FRA10C and FRA10G, and undergo DNA breakage after exposure to fragile site-inducing chemicals. Moreover, exposure of human thyroid cells to these chemicals results in the formation of cancer-specific *RET/PTC* rearrangements. These results provide the direct evidence for the involvement of chromosomal fragile sites in the generation of cancer-specific rearrangements in human cells.
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Introduction

Cancer development can be initiated by the accumulation of various genetic abnormalities that lead to the disregulation of genes involved in various cellular processes. Chromosomal translocations are one of such abnormalities commonly seen in cancer cells. Translocations result in the rearrangement of genetic material, which typically leads to the expression of an oncogenic fusion protein contributing to the neoplastic process (Gasparini *et al*., 2007). To date, there are a total of 705 known recurrent translocations in cancer that involve 459 different gene pairs, and are present in many different types of cancer (Mitelman *et al*., 2008).

In all translocations, the development of breaks in DNA strands must occur. There are various ways in which a cell can acquire these breaks, such as ionizing radiation (Weterings and Chen, 2008). DNA breaks are commonly repaired by two pathways, homologous recombination or non-homologous end joining (Shrivastav *et al*., 2008), but dysfunction of these pathways can contribute to the formation of chromosomal translocations (Gasparini *et al*., 2007). Alternatively, an overwhelming accumulation of DNA breaks could prevent these normally functioning pathways from eliminating all of the breaks, leading to translocation events.

Chromosomal fragile sites are known to contribute to the formation of DNA breaks and are hotspots for sister chromatin exchange (Glover and Stein, 1987), chromosomal translocations, deletions (Glover and Stein, 1988) and viral integrations (Popescu, 2003). Fragile sites are non-random specific loci that are stable under normal conditions, but on certain culture conditions can form visible gaps or breaks in metaphase chromosomes (Durkin and Glover, 2007). Depending on their frequency in the population, fragile sites can be divided into two classes: common and rare. Common fragile sites, which constitute the majority of the two classes, are present in all individuals, and are a normal component of chromosome structure (Glover, 2006). Common fragile sites can be further classified based on their mode of induction, as not all sites are induced by the same compounds, or to the same extent. Aphidicolin (APH) induces expression of the majority of common fragile sites. Other known fragile site-inducing conditions include the addition of 5-bromodeoxyuridine (BrdU), 5-azacytidine and distamycin A and the removal of folic acid (Sutherland, 1991). Also, certain dietary and environmental factors have been shown to contribute to fragile site expression, including caffeine (Yunis and Soreng, 1984), ethanol (Kuwano and Kajii, 1987), hypoxia (Coquelle *et al*., 1998) and pesticides (Musio and Sbrana, 1997). Together, genetic influences

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on fragile site instability, along with external influences from chemical, dietary and environmental factors, suggest a possible role for fragile sites in sporadic cancer formation.

Fragile sites are also known to be late replicating regions of the genome. Delayed DNA replication has been observed in all fragile sites examined to date (Hansen *et al*., 1997; Wang *et al*., 1999; Handt *et al*., 2000; Hellman *et al*., 2000, 2002; Palakodeti *et al*., 2004; Pelliccia *et al*., 2008). Delayed replication at fragile sites is believed to be attributed to the high propensity of DNA sequences to form stable secondary DNA structures (Gacy *et al*., 1995; Usdin and Woodford, 1995; Samadashwily *et al*., 1997; Hewett *et al*., 1998; Mishmar *et al*., 1998; Zlotorynski *et al*., 2003; Zhang and Freudenreich, 2007). Difficulties in passing the replication fork, caused by secondary DNA structure formed within the fragile DNA regions, could result in stalled replication. ATR, a major replication checkpoint protein, is crucial for maintaining fragile site stability (Casper *et al*., 2004), and its inhibition by 2-aminopurine (2-AP) in conjunction with fragile site-inducing chemicals significantly increases common fragile site expression (Casper *et al*., 2002). Therefore, it is suggested that DNA breakage at fragile sites results from delayed replication forks that escape the ATR-mediated checkpoint pathway (Durkin and Glover, 2007).

Many studies point towards the association between fragile sites and formation of cancer-specific translocations (Arlt *et al*., 2006). In a comprehensive survey, we found that 52% of all known recurrent simple chromosomal translocations have at least one gene located within a fragile site, strongly suggesting a potential role for fragile sites in the initiation of translocation events (Burrow *et al*., 2009). Also, Glover and colleagues found that on addition of APH, submicroscopic deletions within *FHIT*, located in the fragile site FRA3B and associated with various human cancers, were detected and resembled those seen in cancer cells (Durkin *et al*., 2008). However, there has been no direct evidence linking breakage at fragile sites to the formation of cancer-causing chromosomal aberrations.

Genes participating in the two main types of *RET/PTC* rearrangements, *RET/PTC1* and *RET/PTC3*, have been mapped to known fragile sites (Burrow *et al*., 2009). *RET/PTC* rearrangements are commonly found in the papillary thyroid carcinomas (PTC), and in all cases result in the fusion of the tyrosine kinase domain of *RET* to the 5′ portion of various unrelated genes (Nikiforov, 2008). In the case of the *RET/PTC1* and *RET/PTC3*, *RET* is fused with *CCDC6* and *NCOA4*, respectively (Santoro *et al*., 2006). These rearrangements result in the expression of a fusion protein possessing constitutive tyrosine kinase activity, which is tumorigenic in thyroid follicular cells (Nikiforov, 2008). Both genes involved in the *RET/PTC3* rearrangement, *RET* and *NCOA4*, are located at 10q11.2 within fragile site FRA10G, a common fragile site induced by APH. The *CCDC6* gene, involved in *RET/PTC1*, is located at 10q21.2 within the fragile site FRA10C, a common fragile site induced by BrdU. Major breakpoint cluster regions for these genes have been identified, and are located within intron 11 of *RET*, intron 5 of *NCOA4* and intron 1 of *CCDC6* (Smanik *et al*., 1995; Nikiforov *et al*., 1999). *RET/PTC* rearrangements are known to be associated with radiation exposure, although most of adult tumors are sporadic and those patients lack the radiation exposure history (Nikiforova and Nikiforov, 2008), implying that other mechanisms should be responsible for DNA breakage and *RET/PTC* formation in most tumors. Clinical studies have shown that *RET/PTC3* rearrangements are common in radiation-induced tumors (Fugazzola *et al*., 1995; Nikiforov *et al*., 1997; Motomura *et al*., 1998). In contrast, sporadic PTC tumors have shown a greater prevalence of *RET/PTC1* rearrangements (Fenton *et al*., 2000), which account for 70% of all *RET/PTC* tumor types (Nikiforova and Nikiforov, 2008). As the participating genes colocalize with fragile sites and there is a well-established association between *RET/PTC* rearrangements and DNA damage induced by ionizing radiation, these rearrangements offer an excellent model to examine directly the role of fragile sites in the formation of cancer-specific chromosomal translocations.

In this study, we show that fragile site-inducing chemicals can create DNA breaks within the *RET/PTC* partner genes and ultimately lead to the formation of *RET/PTC* rearrangements, offering direct evidence for the role of fragile sites in cancer-specific translocations.

Results

Chromosomal disruptions in RET/PTC *gene partners on fragile site induction*

To examine whether chromosomal regions involved in *RET/PTC* rearrangements are part of fragile sites, HTori-3 human thyroid cells were exposed to APH, APH + 2-AP and BrdU + 2-AP. Metaphase spreads of cultured HTori-3 cells were hybridized with fluorescently labeled BAC probes covering the entire genomic sequence of *RET*, *NCOA4* and *CCDC6* (Figure 1). Without exposure to fragile site-inducing chemicals, metaphase chromosomes of HTori-3 cells seemed normal with smooth contours and intact *RET* signal (Figure 1a). With exposure to fragile site-inducing chemicals, the morphology of metaphase chromosomes seemed distorted with irregular surfaces and loss of continuity. After treatment with 0.4 μM APH for 24 h, *RET* was disrupted in 6 ± 0.35% of chromosomes (Figure 1b and Table 1), *NCOA4* was disrupted in 0.62% of chromosomes and no breaks were identified in the *CCDC6* gene (Table 1). The appearance of breaks in *RET* but not in *CCDC6* is consistent with the characteristics of the fragile sites in which each of these genes are located (*RET* located at APH-induced FRA10G and *CCDC6* at BrdU-induced FRA10C). The frequency of breakage observed in *RET* is in agreement with the previously published levels at FRA10G obtained using Giemsa-stained chromosomes, which were found to be at 4.6% on an average after

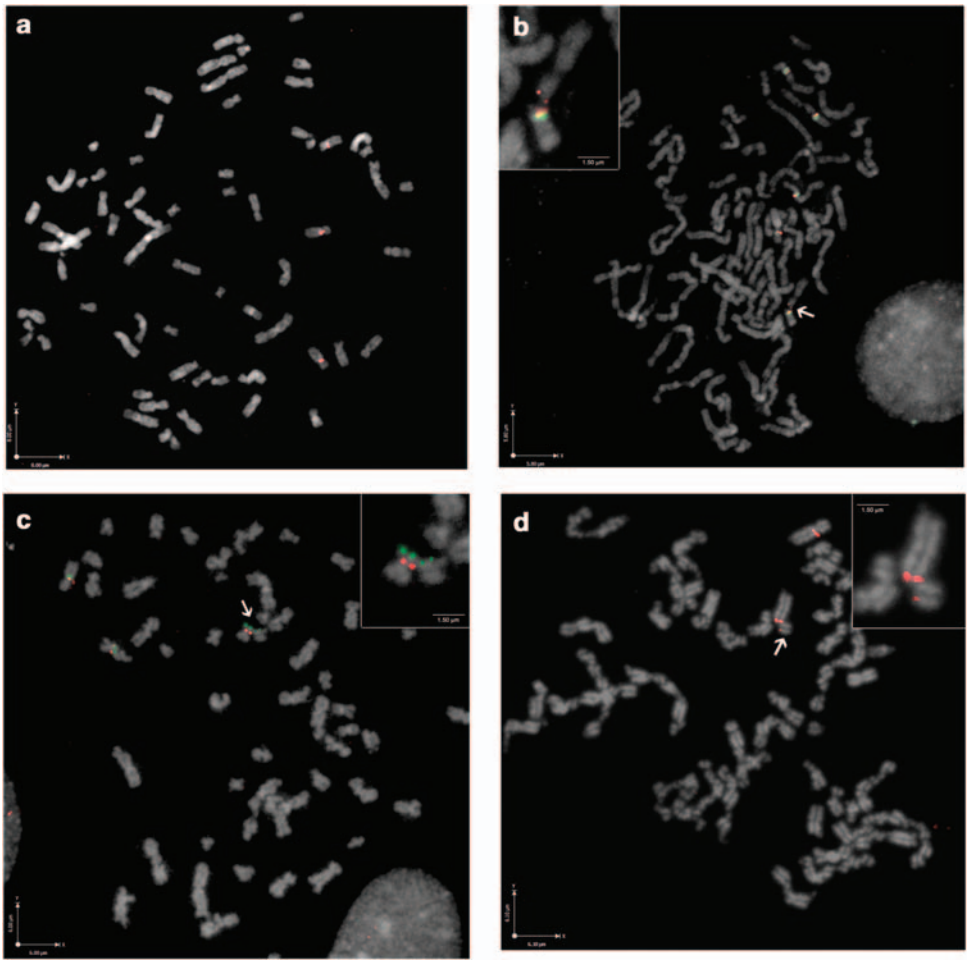


Figure 1 Fluorescence *in situ* hybridization on metaphase chromosomes of HTori-3 cells after treatment with fragile site-inducing chemicals. (**a**) Negative control without treatment showing smooth chromosomes with intact *RET* (red) signal. (**b**) Exposure to APH resulting in irregular chromosome contours and one *RET* signal (red) showing split in the signal whereas four other *RET* signals are intact. Centromeric probe for chromosome 10 is labeled in green. (**c**) Exposure to BrdU + 2-AP resulting in the disruption of *CCDC6* (green) whereas *NCOA4* is intact (red). (**d**) Exposure to APH + 2-AP + BrdU resulting in split in *RET* (red).

Table 1 Percentage of chromosomes showing disruption of *RET*, *NCOA4* and *CCDC6* after exposure to fragile site-inducing agents

	APH	*APH + 2-AP*	*BrdU + 2-AP*
RET	6.00 ± 0.35	5.93 ± 0.52	0.60 ± 0.08
NCOA4	0.62	0.63 ± 0.08	0.60 ± 0.08
CCDC6	0	0.98 ± 0.58	2.72 ± 0.78

Abbreviation: APH, Aphidicolin.

treatment of human skin fibroblasts with 0.2 μM APH for 26 h (Murano *et al*., 1989). After addition of APH and 2-AP, 5.93 ± 0.52% of chromosomes showed breaks in *RET*; 0.63 ± 0.08 % showed breaks in *NCOA4* and 0.98 ± 0.58% showed breaks in *CCDC6*. 2-AP is a general inhibitor of ATR kinase and is known to increase fragile site expression with or without the addition of replication inhibitors like APH (Casper *et al*., 2002). Although breakage in *RET* and *NCOA4* did not change significantly, breakage was now seen in *CCDC6*, consistent with 2-AP action. Treatment with BrdU and 2-AP resulted in 2.72 ± 0.78% of chromosomes showing breaks in *CCDC6* (Figure 1c). However, *RET* and *NCOA4* were each disrupted in 0.6 ± 0.08% of chromosomes after BrdU and 2-AP treatment (Table 1). Increased breakage in *CCDC6* is consistent with its fragile site mode of induction. Also, the level of breakage at *CCDC6* is comparable with previous reports at FRA10C, with DNA breakage ranging from 4–20% after treatment of human blood lymphocytes from 10 patients with 50 mg/l BrdU for 4–6 h (Sutherland *et al*., 1985). The breakage frequency seen in *RET* and *NCOA4* with BrdU and 2-AP treatment is similar to that observed in *CCDC6* after treatment with APH and 2-AP, showing consistency with 2-AP-induced breakage. In concert, these results show directly that chemicals known to result in fragile site breakage cause DNA breaks within genomic sequences of genes participating in *RET/PTC* rearrangements.

Induction of DNA breaks in intron 11 of the RET *gene by APH treatment*

All *RET*/*PTC* rearrangements involve the fusion of the tyrosine kinase domain of *RET*, and the major breakpoint cluster region identified in tumor cells is located within intron 11 (Smanik *et al*., 1995). Although fluorescence *in situ* hybridization (FISH) experiments allowed us to detect breaks occurring within the *RET* gene sequence, whether or not the breaks are located in intron 11 was next examined using ligation-mediated PCR (LM–PCR). HTori-3 cells were treated with APH for 24 h, and the genomic DNAs from both the treated and untreated cells were subjected to primer extension with biotinylated primers that are specific to the regions of interest (see Materials and methods; Supplementary Figure 2). The synthesis reaction terminated at a DNA break to produce a duplex with a blunt end, and the duplex was ligated to a linker. The linker-attached DNAs were then isolated by streptavidin beads, amplified by two rounds of PCR, and visualized by agarose gel electrophoresis (Figure 2). Each lane on the agarose gel represents the DNA breaks isolated from approximately 4000 cells, and each band observed on the gel corresponds to a break found within the region of interest. DNA breaks were observed within intron 11 of *RET* after treatment with APH (Figure 2a) with a frequency of 0.024 ± 0.015 breaks per 100 cells, which was significantly higher than that in the untreated cells (0.004 ± 0.009 per 100 cells, $P = 0.010$; Figure 2b). DNA samples from lanes 1 and 3–6 in Figure 2a (marked with asterisks) were sequenced to determine the location of the induced breakpoints in the *RET* gene (Figure 3). DNA sequencing revealed the breakpoints to be located within intron 11, and at a distance from exon 12 that is consistent with the size of the PCR product observed on the agarose gel in Figure 2a. The locations of these breakpoints were compared with the location of known breakpoints found in PTC tumors containing *RET*/*PTC* rearrangements (Figure 3) (Bongarzone *et al*., 1997; Klugbauer *et al*., 2001). Each induced breakpoint was found to be located near a human tumor breakpoint, with distances ranging from 2–15 base pairs. It is important to note that these induced breakpoints were detected before a rearrangement event, whereas the breakpoints found in tumors have been identified after a rearrangement event has occurred. In most cases, small modifications, such as deletions and insertions of 1–18 nucleotides, have been observed surrounding the fusion points in human tumors. These results confirm that the exposure of thyroid cells to APH induces the formation of DNA breaks within the major breakpoint cluster region found in the *RET* gene, and these induced breakpoints are located close to known breakpoints found in human tumors.

DNA breaks were also examined within FRA3B after APH treatment. FRA3B is the most inducible fragile site

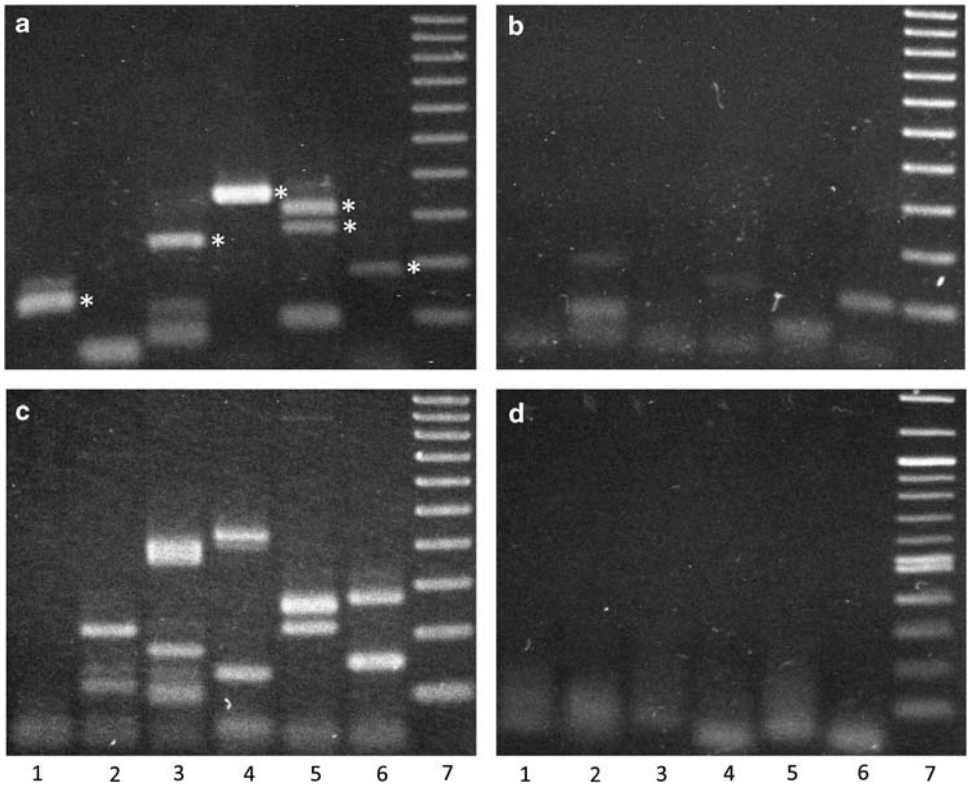


Figure 2 LM–PCR detection of breaks formed in HTori-3 cells after treatment with APH. LM–PCR detection of DNA breaks formed in HTori-3 cells at intron 11 of *RET* (**a**), the fragile site FRA3B (**c**) and the non-fragile 12p12.3 region (**d**) after treatment with APH. The same reaction was carried out as in (**a**) for intron 11 of *RET*, but using DNA from cells without APH treatment (**b**). Last lane of each gel is a 100 bp molecular weight ladder. Bands below 100 bp correspond to primer dimers. Asterisks mark DNA fragments that were sequenced, and results are shown in Figure 3.

a

b

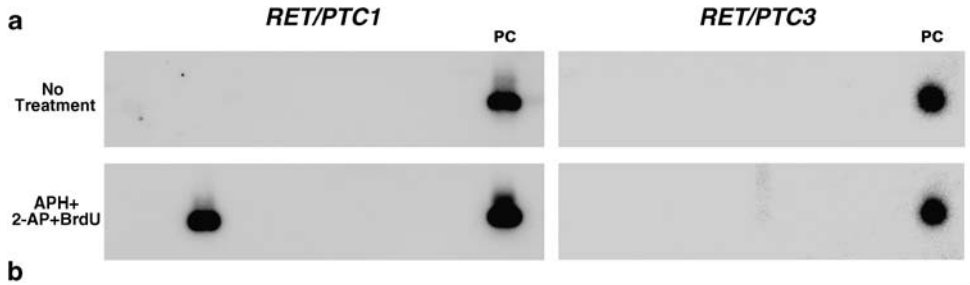
Lane	Induced Breakpoint Distance from 5′ end of RET-R1 primer (bp)	Distance of Induced Breakpoint from Closest Patient Breakpoint (bp)
1	161	3
3	230	6
4	370	15
5	325	2
	298	15
6	174	10

Figure 3 Location of breakpoints within intron 11 of *RET* induced by treatment with APH. (**a**) DNA samples from lanes 1, and 3–6 in Figure 2a (marked with asterisks) were sequenced, and six breakpoints are identified and indicated by black arrowheads. The locations of known breakpoints found in tumors containing *RET*/*PTC* rearrangements are indicated by gray arrowheads (Bongarzone *et al*., 1997; Klugbauer *et al*., 2001). The gray arrow corresponds to the RET-7 primer with a dual biotin label (gray circles), which is annealed to exon 12 of the *RET* gene. The black solid and dashed arrows correspond to the RET-R1b and RET-R1 nested PCR primers, respectively. The sequence of intron 11 is italicized. (**b**) The distance of each induced breakpoint from the 5′ end of the RET-R1 primer and the nearest patient tumor breakpoint was listed.

in the human genome and contains *FHIT*, a gene involved in several cancers, where microscopic deletions have been observed after treatment with APH (Wang *et al*., 1999; Durkin *et al*., 2008). Intron 4 of the *FHIT* gene, a major region of high instability in various tumors and APH-treated cells (Boldog *et al*., 1997; Corbin *et al*., 2002), was examined for DNA breaks. DNA breaks were detected within intron 4 of *FHIT* on APH treatment (Figure 2c) at a frequency of 0.036 ± 0.020 breaks per 100 cells, confirming that indeed the APH treatment can induce fragile site breakage. An increased number of breaks were observed within FRA3B in comparison to *RET*, which is consistent with FRA3B being the most active fragile site in the genome. A non-fragile region, 12p12.3 (Zlotorynski *et al*., 2003) and the *G6PD* gene, within FRAXF (a rare folate-sensitive fragile site not induced by APH), were examined after treatment with APH, and in contrast to *RET* and FRA3B, no DNA breaks were observed within the 12p12.3 region (Figure 2d) or in exon 1 of *G6PD* (Supplementary Figure 3). The absence of breaks in 12p12.3 and *G6PD* suggests that the DNA breaks observed within *RET* and FRA3B after exposure to fragile site-inducing chemicals are because of their fragile nature in response to APH.

Generation of RET/PTC *rearrangements after treatment with fragile site-inducing chemicals*

To test for the induction of *RET*/*PTC* rearrangement after exposure to fragile site-inducing chemicals, HTori-3 cells were treated with APH and 2-AP for 24 h with the addition of BrdU for 5 h. These treatment conditions were chosen because they have been previously established to be optimal for the induction of fragile sites FRA10C and FRA10G (Sutherland *et al*., 1985; Murano *et al*., 1989). To confirm breakage in the genes after exposure, metaphase spreads were made and chromosomes were scored for disruption of the probe (Figure 1d). The breakage in the probes for *RET*, *NCOA4* and *CCDC6* were 7.47, 1.15 and 2.87%, respectively. The mRNA was then isolated and used in RT–PCR for detection of *RET*/*PTC1* and *RET*/*PTC3* formation. To assure that a cell with the rearrangement would be detected, 1×10^6 cells in a 10 cm culture dish were divided among 30 culture dishes 24 h post-exposure. Therefore, each well received no more than 3×10^4 cells, and if a dish contained only one cell with *RET*/*PTC*, it would constitute one part in 3×10^4, a fraction within the limit of detection (Caudill *et al*., 2005). No *RET*/*PTC* rearrangement was detected without any treatments in five independent experiments (Figure 4), indicating an extremely low level of spontaneous generation of *RET*/*PTC* in this human cell line and the absence of contamination. Similarly, no *RET*/*PTC* rearrangement was detected using the same experimental approach in HTori-3 cells in four independent experiments in a study reported by Caudill *et al*. (2005). Exposure to a combination of APH, 2-AP and BrdU resulted in the generation of *RET*/*PTC1*, with five total events identified in five independent experiments, each assaying 10^6 cells (incidence of 2, 1, 2, 0, 0 events per 10^6 cells) (Figure 4b). However, no *RET*/*PTC3* rearrangements were identified. Representative RT–PCR blots are shown in Figure 4a. Statistical analysis revealed a significant difference in the incidence of *RET*/*PTC1* induction between untreated cells (zero events) and cells treated with fragile site-inducing agents (five total events) ($P = 0.027$). These results show that the exposure of thyroid cells to fragile site-inducing chemicals can lead to the formation of a carcinogenic *RET*/*PTC* rearrangement.



b

	Experimental Replicates	*RET/PTC1* events	*RET/PTC3* events
No Treatment	5	0	0
APH+2-AP+BrdU	5	5	0

Figure 4 Detection of *RET*/*PTC* rearrangements in HTori3 cells after treatment with fragile site-inducing chemicals. (**a**) Detection of *RET*/*PTC* rearrangements in representative RT–PCR experiment after exposure to APH + 2-AP + BrdU. (PC, positive control). (**b**) Number of rearrangement events detected in untreated cells and cells exposed to APH + 2-AP + BrdU. Five independent experiments were carried out for each treatment, and each experiment analyzed 10^6 cells.

Discussion

Chromosomal rearrangements contribute to the development of many types of human tumors. Therefore, it is critical to understand the mechanisms of chromosomal rearrangements in cancer cells. Here, we showed that DNA breakage at fragile sites FRA10C and FRA10G under fragile site-inducing conditions initiates and leads to the generation of *RET*/*PTC1* rearrangement, which is known to contribute to PTC development. To our knowledge, this is the first demonstration that a cancer-specific rearrangement can be produced in human cells by inducing DNA breaks at fragile sites. Interestingly, only *RET*/*PTC1* rearrangements were observed, and no *RET*/*PTC3* rearrangements were identified. Although breakage was seen within *NCOA4*, the *RET*/*PTC3* partner gene, the frequency of breakage was lower when compared with *RET* and *CCDC6*. *NCOA4* breakage remained relatively constant with each combination of fragile site-inducing chemicals, and was about 10-fold lower than the breakage observed within *RET,* and about 4.5-fold below the level found in *CCDC6*. The lower incidence of breakage within *NCOA4* could contribute to the lack of *RET*/*PTC3* rearrangement events. Also, clinical studies have revealed that *RET*/*PTC3* rearrangements are frequent in radiation-induced tumors (Fugazzola *et al*., 1995; Nikiforov *et al*., 1997; Motomura *et al*., 1998), whereas *RET*/*PTC1* rearrangements are more commonly seen in sporadic tumors (Fenton *et al*., 2000). Our observation of *RET*/*PTC1* rearrangement, but not *RET*/*PTC3* rearrangement, generated by fragile site induction, further supports the idea that sporadic PTC tumors may result from breakage at fragile sites. It is known that specific environmental and food toxins (such as caffeine, alcohol and tobacco) (Yunis and Soreng, 1984; Kuwano and Kajii, 1987), and other stress factors (such as hypoxia) (Coquelle *et al*., 1998) can induce fragile sites. Therefore, our results suggest that these exogenous factors may contribute to the occurrence of chromosomal rearrangements, and therefore cancer initiation in human populations, by a mechanism of DNA breakage at fragile sites.

To show that fragile site-inducing chemicals can cause DNA breaks at *RET*/*PTC* participating genes, FISH analysis of chromosome 10, and LM–PCR analysis at the nucleotide level of the *RET* gene were performed. Using FISH, we showed that on exposure of human thyroid cells to fragile site-inducing chemicals, chromosomal breaks are formed within the *RET* and *CCDC6* genes. *RET* and *CCDC6* are located respectively within the APH and BrdU-induced fragile sites, and show breakage only after the addition of APH or BrdU, accordingly. These results not only show that the fragility is indeed present within the genes involved in *RET*/*PTC* rearrangements, but also underline the specificity of fragile site induction that was observed in these regions. Although 2-AP addition is known to increase overall chromosomal breakage and fragile site FRA3B expression (Casper *et al*., 2002), no significant increase in breakage at *RET* and *NCOA4* genes was noted in HTori-3 cells, indicating its weaker influence on the FRA10G site. Furthermore, the addition of 2-AP in combination with APH resulted in the appearance of breaks within *CCDC6*, whereas its combination with BrdU resulted in breaks within *RET* and *NCOA4*. This nonspecific effect of 2-AP on induction of DNA breaks at fragile sites is in agreement with its ability to inhibit ATR protein, which provides a key maintenance role in fragile site stability.

The DNA breaks generated in *RET* after exposure to APH were confirmed to be located within intron 11, which is the breakpoint cluster region identified in thyroid tumors, whereas untreated cells showed little to no breaks. These breaks are further confirmed to be fragile in nature, when comparing the formation of breaks within FRA3B, 12p12.3 and *G6PD* regions. FRA3B, the most inducible fragile site in the human genome (Wang *et al*., 1999; Durkin *et al*., 2008), showed DNA breaks after treatment with APH (Figure 2c), whereas 12p12.3, a non-fragile region, and the *G6PD* gene, located within a rare folate-sensitive fragile site, showed no DNA breakage with the same treatment (Figure 2d and Supplementary Figure 3b). Together with cytogenetic analysis, these results show that fragile site-inducing chemicals can generate breaks within *RET*

and *CCDC6* genes, which could result in the formation of cancer-causing *RET/PTC1* rearrangement.

The induction rate of *RET/PTC* rearrangement by fragile site-inducing chemicals was four magnitudes lower than the frequency of chromosomal breaks observed in *RET* and *CCDC6* genes. DNA breaks, a serious threat to genome stability and cell viability, can trigger DNA repair pathways, including homologous recombination or non-homologous end joining (Shrivastav *et al*., 2008). The action of these pathways ensures proper repair of DNA breaks, and prevents the deleterious consequences of such breakage. However, some (small number of) DNA breaks escaping the repair pathways will ultimately result in large-scale chromosomal changes, such as *RET/PTC* rearrangement.

This study provides important information about the mechanisms of formation of carcinogenic chromosomal rearrangements in human cells. In addition, it establishes an experimental system that will allow for testing the role of specific environmental substances, dietary toxins and other stress factors in the generation of chromosomal rearrangements and tumor initiation.

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. They are characterized as immortalized, partially transformed, differentiated cells having three copies of chromosome 10 with intact *RET*, *NCOA4* and *CCDC6* loci and preserve the expression of thyroid differentiation markers, such as thyroglobulin production and sodium iodide symporter, as we reported previously (Caudill *et al*., 2005). The cells were purchased from the European Tissue Culture Collection and grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum.

Fragile site induction

HTori-3 cells (1×10^6) were plated in 10 cm culture dishes and after 16 h were exposed to APH for 24 h (0.4 μM) or APH and 2-AP (2 mM) (Casper *et al*., 2002). When desired, cells were treated with BrdU (50 mg/l) for 5 h, in addition to 2-AP and/or APH for 24 h. For DNA breaksite detection, 5×10^5 cells were plated in 10 cm culture dishes and treated in the same manner as above with 0.4 μM APH.

Metaphase chromosome preparation

HTori-3 cells exposed to various chemicals were treated with 0.1 μg/ml of Colcemide for 2 h before harvesting. Cells were incubated in hypotonic solution (0.075 M KCL), fixed in multiple changes of methanol:acetic acid (3:1) and dropped onto moistened slides to obtain metaphase spreads. Slides were aged overnight and pretreated with RNase before proceeding for hybridization.

Probes for FISH

BAC clones RP11-351D16 (*RET*), RP11-481A12 (*NCOA4*), RP11-435G3 and RP11-369L1 (*CCDC6*) were obtained from BAC/PAC Resources, Children's Hospital, Oakland, CA, USA. BAC clone RP11-481A12 containing the *NCOA4* gene was subcloned into fosmid vector after cutting with restriction enzymes (Epicenter). A mixture of subcloned probes (SC10, SC19) containing 70 kb of the *NCOA4* gene and its flanking regions was used as a probe for *NCOA4*. The probes were labeled by nick translation using Spectrum Green-dUTP, Spectrum Orange-dUTP or Spectrum Red-dUTP (Abbott Laboratories, Des Plaines, IL, USA). Hybridization was performed as previously described (Ciampi *et al*., 2005). On average, 150 chromosomes were scored for breaks in the *RET*, *NCOA4* and *CCDC6* probes for each condition.

DNA breaksite mapping by LM–PCR

To detect DNA breaks within intron 11 of *RET* induced by APH, a 5′-biotinylated primer RET-7 corresponding to the *RET* at the 5′ end of exon 12 (the gray arrow in Figure 3a) was used to extend into intron 11. For first and second rounds of nested PCR primers RET-R1b and RET-R1 were used, respectively. To isolate the DNA breaks, a duplex DNA linker LL3/LP2 was used as described (Kong and Maizels, 2001) as well as the corresponding linker specific primers LL4 and LL2 (Supplementary Figure 2). For FRA3B, the biotinylated primer FRA3B-20 was used to allow identification of break sites occurring at intron 4 of the *FHIT* gene, which contains major clusters of APH-induced breakpoints in FRA3B (Boldog *et al*., 1997; Corbin *et al*., 2002), and primers FRA3B-9 and FRA3B-23 were used in first and second rounds of nested PCR, respectively. For detection of breaks within the 12p12.3 region, the biotinylated primer 12p12.3-1 and primers 12p12.3-2 and 12p12.3-3 were used. For detection of breaks within exon 1 of *G6PD*, the biotinylated primer G6PDF3 and primers G6PDF and G6PDF2 were used. Sequence of linkers and PCR primers is described in the Supplementary Figure 1.

DNA breaksite mapping was performed as described (Kong and Maizels, 2001) with modifications (Supplementary Figure 2). Genomic DNA was isolated from HTori-3 cells with or without APH treatment. Primer extension was performed using 200 ng of DNA at 45 °C, and the DNA breaks were isolated through ligation of the LL3/LP2 linker, and then using streptavidin beads. Amplification of these DNA breaks was achieved by nested PCR of the extension–ligation products. The final PCR products were resolved by electrophoresis on a 1.3% agarose gel. Each band observed on the gel corresponds to a break isolated within the region of interest. To confirm the bands observed were located within intron 11 of *RET*, the PCR products were sequenced. The exact breakpoint sites were determined from the sequencing results by identifying the nucleotide adjacent to the LL3/LP2 linker sequence.

Detection of RET/PTC *rearrangements*

Upon treatment with fragile site-inducing agents for 24 h, cells were split into 30 6 cm culture dishes at a density of approximately 3×10^4 cells per dish and grown for 3–4 days. To sustain growth for 9 days, cells were transferred to 10 cm culture dishes for 4–5 days after seeding into 6 cm dishes. RNA was extracted from each culture dish using a Trizol reagent (Invitrogen). Then, mRNA was purified using the Oligotex mRNA minikit (Qiagen, Valencia, CA, USA). RT–PCR was performed using a Superscript first strand synthesis system kit and random hexamer priming (Invitrogen,). PCR was performed to simultaneously detect *RET/PTC1* and *RET/PTC3* rearrangement using primers *RET/PTC1* forward, *RET/PTC3* forward, and common reverse (Supplementary Figure 1). As positive controls, cDNA from *RET/PTC1*-

positive TPC-1 cells and *RET/PTC3* positive tumor sample were used. Ten micro liter 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* and *RET/PTC3* (Supplementary Figure 1). Evidence of *RET/PTC* rearrangement in the cells from a given flask was scored as one *RET/PTC* event. All statistics performed using one-tailed Student's *t*-test.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Supplementary Figure 1

Linker and Primer Sequences for LM-PCR

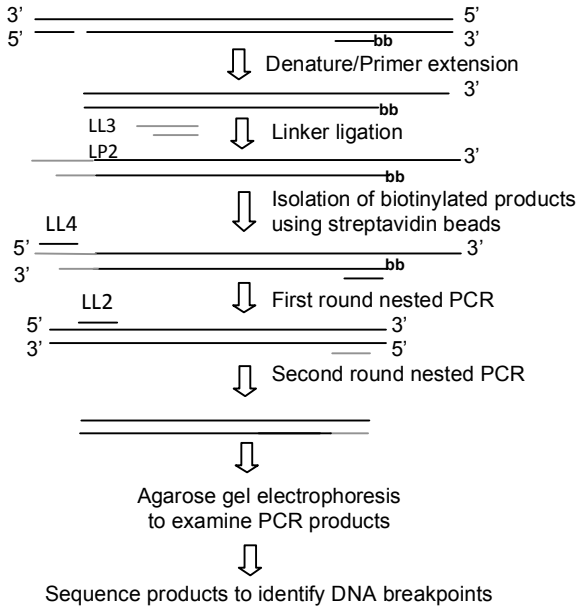
LL3	5’-CGAGTTCAGTCCGTAGACCATGGAGATCTGAATTC-3’
LP2	5’-GAATTCAGATCTCC-3’
LL4	5’-CGAGTTCAGTCCGTAGAC-3’
LL2	5’-GTAGACCATGGAGATCTGAAATTC-3’
RET-7	5’- BBCAGCATCTTCACGGCCACCGTGG-3’ (B, biotin)
RET-R1b	5’- TACCCTGCTCTGCCTTTCAGATGG-3’
RET-R1	5’-AGTTCTTCCGAGGGAATTCC-3’
FRA3B-20	5’- BBCCTATCTGACGACTTCAC-3’ (B, biotin)
FRA3B-9	5’- GAAAGCATAAAGTGTGGC-3’
FRA3B-23	5’- TAACTGCTTATTTTTCCGATGT-3’
12p12.3-1	5’- BBTTTTCTTGACTAGTCTAACCAGAT-3’ (B, biotin)
12p12.3-2	5’- TTTCACTTGTATTGATCTCCTTCAT-3’
12.12.3-3	5’- TTTCCACTGTTTGCCGCATTAT-3’
G6PDF3	5’-BBAGTAAAAACACAAGCCCCGCCCC-3’ (B, biotin)
G6PDF	5’-TAGGGCCGCATCCCGCTCCGGAGAGAAGTCT-3’
G6PDF2	5’-GGCCACTTTGCAGGGCGTCA-3’

PCR primers for Detection of *RET/PTC* Rearrangements

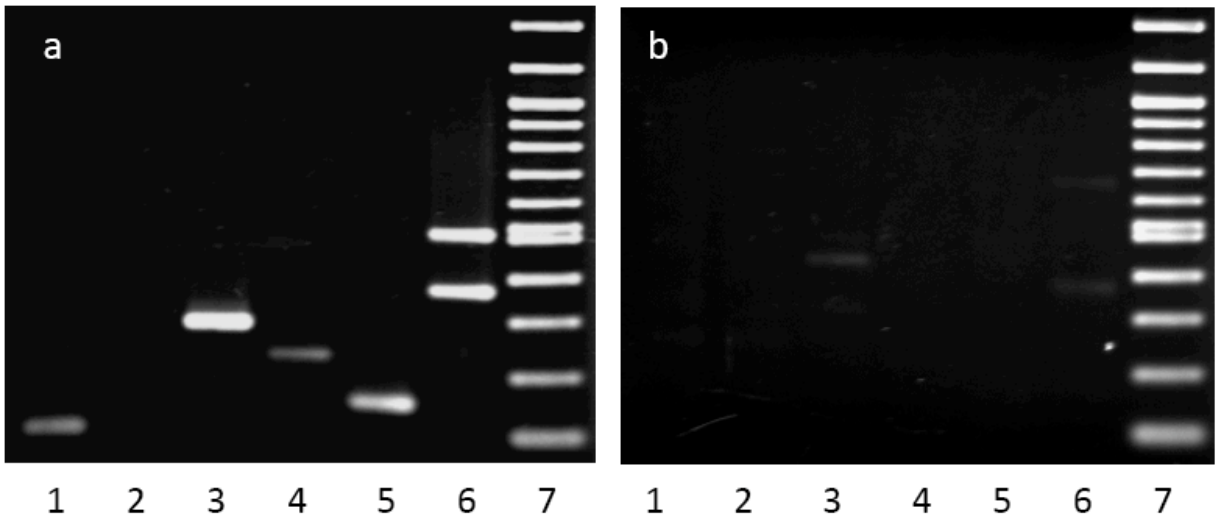
RET/PTC1 forward	5'-CAAGAGAACAAGGTGCTGAAG-3'
RET/PTC3 forward	5'-CGGTATTGTAGCTGTCCCTTTC-3'
common reverse	5'-GCAGGTCTCGAAGCTCACTC-3'

^{32}P-labeled oligonucleotide probes

RET/PTC1	5'-CGTTACCATCGAGGATCCAAA-3'
RET/PTC3	5'-GAACAGTCAGGAGGTCCAA-3'



Supplementary Figure 2. DNA breaksite mapping by LM-PCR. Genomic DNA was isolated from HTori-3 cells with or without APH treatment, and was denatured and then annealed to a biotinylated primer specific for the region of interest. Primer extension was carried out with DNA Sequenase, and the reaction terminates at a DNA break. DNA breaks were isolated through ligation of the LL3/LP2 linker, and recovered by streptavidin beads. Amplification of these DNA breaks was achieved by nested PCR of the extension-ligation products. The final PCR products were resolved by agarose gel electrophoresis. Each band observed on the gel corresponds to a break found within the region of interest. The exact breakpoint sites were determined by DNA sequencing of the PCR products, and by identifying the nucleotide adjacent to the LL3/LP2 linker sequence.



Supplementary Figure 3. LM-PCR detection of breaks formed in HTori-3 cells after treatment with APH. LM-PCR detection of DNA breaks formed in HTori-3 cells at intron 11 of *RET* (**a**) and exon 1 of *G6PD* (**b**) after treatment with APH. Last lane of each gel is a 100 bp molecular weight ladder.