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Advances in Brief

Translocation t(10;14)(q11.2;q22.1) Fusing the *Kinectin* to the *RET* Gene Creates a Novel Rearranged Form (PTC8) of the *RET* Proto-Oncogene in Radiation-induced Childhood Papillary Thyroid Carcinoma[1]

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Abstract

Evaluation of 20 cases of radiation-induced childhood papillary thyroid carcinoma using fluorescence *in situ* hybridization demonstrated the presence of clonal translocations affecting the *RET* locus. Semiquantitative reverse transcription-PCR indicated overexpression of the *RET* tyrosine kinase (TK) domain in four cases. In two cases, the *RET* rearrangements PTC6 and PTC7 were identified and assigned to balanced translocations t(7;10)(q32;q11.2) and t(1;10)(p13;q11.2), respectively. In one case with a balanced translocation t(10;14)(q11.2;q22.1), 5′ rapid amplification of cDNA ends revealed a novel type of *RET* oncogenic activation (PTC8), arising from a fusion of the 5′ part of the *kinectin* (*KTN1*) gene to the TK domain of the *RET* gene. The presence of coiled-coil domains in the resulting ktn1/ret fusion protein suggests ligand-independent dimerization and thus constitutive activation of the ret TK domain.

Introduction

Thus far, several different forms of *RET* proto-oncogene activation have been reported in PTCs[3] from children in areas of Belarus exposed to fallout from the Chernobyl reactor accident. In all of these cases, the TK domain of the *RET* proto-oncogene is fused to 5′ end sequences of different genes constitutively expressed in follicular cells of the thyroid. As a consequence, active chimeric forms of the *RET* proto-oncogene occur, which are responsible for the generation of fusion proteins exhibiting coiled-coil domains that allow dimerization and thus ligand-independent activation of the cytoplasmic ret TK domain (1–8). The chromosomal mechanisms generating the oncogenic versions of PTC1, PTC3, and PTC4 have been identified as paracentric inversions on 10q with the activating genes *H4* and *ELE1* located at 10q21 and 10q11.2, respectively (7, 9). The PTC2 oncogenic form is caused by a balanced translocation t(10;17)(q11.2;q23) fusing the TK domain of the *RET* proto-oncogene with the regulatory subunit RIα of the c-AMP-dependent protein kinase (9). The PTC5 form is reported to be the result of a chromosomal rearrangement fusing the 5′ end of a gene designated *RFG5* with the *RET* TK domain (3). In the PTC6 rearrangement, the *RET* TK domain is fused to the *hTIF1* gene (4). In the PTC7 activating form, the *RET* TK domain is fused to a *hTIF1*-related gene designated as *RFG7* (4). Recently, a novel rearrangement was found in a sporadic PTC in which the *RET* gene was joined to the *ELKS* gene because of a chromosomal translocation t(10;12)(q11;p13) (10).

In the present study, we have re-evaluated 20 radiation-induced childhood PTCs from a previous investigation (8) that were negative for PTC1–4 types of *RET* rearrangements. These cases were screened for additional *RET* rearrangements in interphase and metaphase cells using FISH with *RET*-specific YAC DNA probes (11). In one case, a novel oncogenic *RET* rearrangement could be identified and designated PTC8. It is caused by a balanced translocation t(10;14)(q11.2;q22.1) fusing the *RET* TK domain to the *KTN1* gene (12, 13). In two additional cases, the recently described *RET* rearrangements PTC6 and PTC7 were detected and could be assigned to balanced translocations t(7;10)(q32;q11.2) and t(1;10)(p13;q11.2), respectively.

Materials and Methods

Tumor Specimens. Thyroid tumor tissues were obtained from Belarussian children who underwent surgery at the Department of Surgery, Medical High School of Minsk. All patients lived in areas contaminated by radioiodine from the Chernobyl reactor accident (8).

Tissue Culture and Chromosome Preparation. Primary cell culture and chromosome preparation were performed as described previously (14). In brief, disaggregated tissues were seeded directly onto glass slides, and chromosome preparations were carried out after an *in vitro* culture of cells for 8–21 days. The epithelial nature of cultured cells was assessed by immunocytochemical staining of anticytokeratin (AE1/AE3; Boehringer Mannheim). For FISH analysis, slides were aged for 7 days at 37°C and stored at −20°C under nitrogen atmosphere until use.

FISH with *RET*-specific YAC DNA Probes. The YAC DNA probes used in the present study were selected as described previously (11). The YAC clones 313F4 and 214H10 map proximal to and include the *RET* gene locus, whereas clone 55A10 contains DNA sequences distal to *RET*. Total yeast DNA was extracted according to standard procedures and was labeled with digoxigenin-11-dUTP (55A10) or biotin-16-dUTP (214H10 and 313F4) by nick translation. Hybridization and detection of fluorescence signals was performed as described previously (15).The cells were analyzed with a Zeiss Axioplan 2 fluorescence microscope equipped with filter sets for 4′,6-diamidino-2-phenylindole, FITC, and tetramethylrhodamine isothiocyanate. For each investigated case, at least 100 images (interphase cells and metaphase spreads) were acquired using the ISIS3/V. 3.04 software (Metasystems, Altlussheim). The hybridization efficiency of the selected probe combination to the *RET* locus was tested on cultivated normal thyroid epithelial cells. Cells from a short-term primary culture derived from a thyroid tumor with a PTC1 rearrangement, previously proven by RT-PCR and direct sequencing (8), were used to evaluate the suitability of this set of probes to detect *RET* rearrangements in interphase nuclei and metaphase spreads.

mRNA Isolation, RT-PCR, and Semiquantitative RT-PCR. Poly(A)$^+$ mRNA was extracted from thyroid tumors using a Micro-Fast Track mRNA Isolation kit (Invitrogen, Leek, the Netherlands). Reverse transcription was performed with a cDNA Cycle kit (Invitrogen), and RT-PCR was carried out using specific primers (PTC5aV/retc2, PTC6bV1/retc5, and PTC7V/retc2;

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[3] The abbreviations used are: PTC, papillary thyroid carcinoma; YAC, yeast artificial chromosome; TK, tyrosine kinase; FISH, fluorescence *in situ* hybridization; RT-PCR, reverse transcription-PCR; RACE, rapid amplification of 5′ cDNA ends.

Table 1 *Position of selected primers*

Primer	Gene	Position[a]	Sequence	Reference
retc1	c-*RET*	2080–2099	TGGGAATTCCCTCGGAAGAA	(1)
retc2	c-*RET*	2295–2314	TGCAGGCCCCATACAATTTG	(1)
retc5	c-*RET*	2206–2225	GAGGCGTTCTCTTTCAGCAT	(4)
tm1	c-*RET*	1853–1872	CTGTCCTCTTCTCCTTCATC	(1)
RETSP2	c-*RET*	2206–2223	GGCGTTCTCTTTCAGCAT	This study
RETSP3	c-*RET*	2161–2182	CTGCTCTGCCTTTCAGATGGAA	This study
PTC5aV	*RFG5*	1403–1422	TGGAAGAACTTCGGCATGAG	(3)
PTC6bV1	*hTIF1*	1317–1336	GAATTCACAGCCACCAAGTG	(4)
PTC7V	*RFG7*	2527–2546	CTACTTAGCTTTCCAAGTGG	(4)
KTN1F	*KTN1*	2717–2739	ACAGGGAAGTGGTTACAGGATCT	This study
KTN1R	*KTN1*	3189–3210	GGGACAGACACCTTTGGAAATA	This study

[a] Position according to current sequences of EMBL database: *RET* (accession no. X12494); *RFG5* (accession no. AJ132949); *TIF1* (accession no. NM003852); *RFG7* (accession no. AJ132948); and *KTN1* (accession no. L25616).

Table 1) for the recently detected *RET* rearrangements PTC5–7. In cases that failed to show expression of known ret/PTC chimeric transcripts, semiquantitative RT-PCR was performed as described (1) using primers tm1, retc1, and FlTC-labeled retc2 (Table 1). The generated PCR products TM/TK (462 bp) and TK (235 bp) were semiquantitatively analyzed on an automatic sequencer (ALF; Pharmacia).

5′ RACE. Rapid amplification of unknown 5′ cDNA ends was performed using the 5′ RACE system (Life Technologies, Inc.) according to the manufacturer's recommendations. In brief, first-strand cDNA was synthesized from poly(A)$^+$ mRNA using a *RET*-specific reverse primer (retc2; Table 1). A homopolymeric tail was then added to the 3′-end of the resulting cDNA using terminal deoxynucleotidyl transferase and dCTP. PCR amplifications were accomplished using the provided forward primers and *RET*-specific reverse primers RETSP2 and RETSP3 (Table 1) containing uracil DNA glycosylase cloning sequences. PCR products were cloned into the pAMP1 vector using the CLONEAMP uracil DNA glycosylase cloning method (Life Technologies, Inc.). SP6 and T7 promoter primers were used for sequencing of selected clones containing the unknown 5′-end of chimeric cDNA representing novel *RET* oncogenic rearrangements.

Results and Discussion

Validation of YAC Probes. FISH on cultivated normal thyroid epithelial cells with unrearranged *RET* loci revealed two yellow signals or tightly colocalized red and green signals in metaphase spreads (Fig. 1*A*) and in interphase nuclei (Fig. 1*B*). In metaphase (Fig. 1*C*) and interphase (Fig. 1*D*) cells from a thyroid tumor with a previously proven PTC1 rearrangement, the expected paracentric inversion, could be clearly demonstrated by the presence of colocalized signals on normal chromosome 10 but split red and green signals on the affected chromosome 10.

Detection of Novel *RET* Rearrangements. Twenty PTC1–4 negative cases from our previous RT-PCR study (8) for which primary cell cultures were available were re-evaluated for novel *RET* rearrangements using *RET*-specific YAC DNA probes. The molecular-cytogenetic analysis in interphase nuclei revealed in each of four cases (S253, S271, S284, and S299) colocalized signals accompanied by split signals. The larger physical distances compared with those observed for the intrachromosomal PTC1 paracentric inversion were regarded as an indication for the existence of interchromosomal rearrangements affecting the *RET* locus (Fig. 1*F*). Actually, translocations could be confirmed on metaphase spreads (Fig. 1*E*) and cytogenetically characterized in detail using the 4′,6-diamidino-2-phenylindole banding pattern in combination with FISH [S253, t(10;15)(q11.2;q22); S271, t(10;14)(q11.2;q22.1); S284, t(1;10)(p13;q11.2); and S299, t(7;10)(q32;q11.2)]. RT-PCR using specific primers (Table 1) for PTC5–7 rearrangements and subsequent direct sequencing demonstrated in cases S284 and S299 the presence of PTC7 and PTC6 transcripts, respectively (Fig. 2*A*), whereas in cases S253 and S271, none of these recently identified *RET* rearrangements were found. Interestingly, the balanced translocation t(1;10)(p13;q11.2) generating the PTC7 rearrangement has been reported previously in a sporadic papillary thyroid carcinoma (16). In S253 and S271, semiquantitative RT-PCR analysis of the simultaneously generated PCR fragments TM/TK and TK demonstrated a clear quantitative shift toward the TK fragment (Fig. 2*C*). This strongly indicates that the respective balanced translocations represent novel types of *RET* oncogenic rearrangements.

Identification of the *RET* Fused Gene. The 5′ RACE technique was performed to amplify the unknown 5′ ends of rearranged *RET* cDNA from tumor samples S253 and S271. The resulting cDNA fragments were cloned and sequenced. Comparison of the obtained sequences with the EMBL database using the BLAST program showed that in case S271, a 5′ unrelated sequence was fused to the *RET* cDNA sequence. This sequence was identical to the 5′ part of the *KTN1* gene (Ref. 17; Fig. 3), which has been mapped by FISH to chromosomal band 14q22.1 (18). To our knowledge, this is the first report on the involvement of *KTN1* in the development of human tumors. In case S253, studies are in progress to determine the respective ret-fused gene.

Confirmation of 5′ RACE Results. To confirm the expression of *KTN1*/*RET* fusion mRNA in tumor S271, we performed RT-PCR with appropriate primers (KTN1F/RETSP3; Table 1) that generate a PCR fragment spanning the fusion region. We successfully amplified the expected PCR product *KTN1*/*RET* (331 bp) if cDNA from this tumor was used as a template but not if cDNA from the corresponding nontumorous material was used (Fig. 2*B*). Finally, the use of *KTN1*-specific primers (KTN1F/KTN1R; Table 1) resulted in amplification of the expected PCR fragment (494 bp) in the corresponding nontumorous material (Fig. 2*B*), demonstrating constitutive expression of *KTN1* in thyroid epithelium. The authenticity of RT-PCR products was confirmed by direct sequencing.

Putative Role of the *KTN1/RET* Fusion Protein in Development of PTCs. Kinectin is a cytoplasmic-oriented vesicle membrane-anchored protein that interacts with the molecular motor kinesin, promoting the kinesin-dependent organelle movement along microtubules. The predicted open reading frame encodes for a protein of 156 kDa molecular mass, which contains an NH_2-terminal transmembrane domain and two COOH-terminal leucine zipper motifs (12). Analysis of the amino acid sequence predicted the formation of α helical domains within a large region between residues 327 and 1362 (12). The presence of heptad repeats (13, 17) in the helical domain regions is highly indicative for the formation of coiled-coil structures, suggesting that kinectin can form dimers. The dimerization capacity of kinectin is further supported by the presence of the above-mentioned leucine zipper motifs located between amino acid residues 934–962 (12).

The comparison of the kinectin amino acid sequence (12) with the predicted ktn1/ret fusion protein sequence revealed that amino

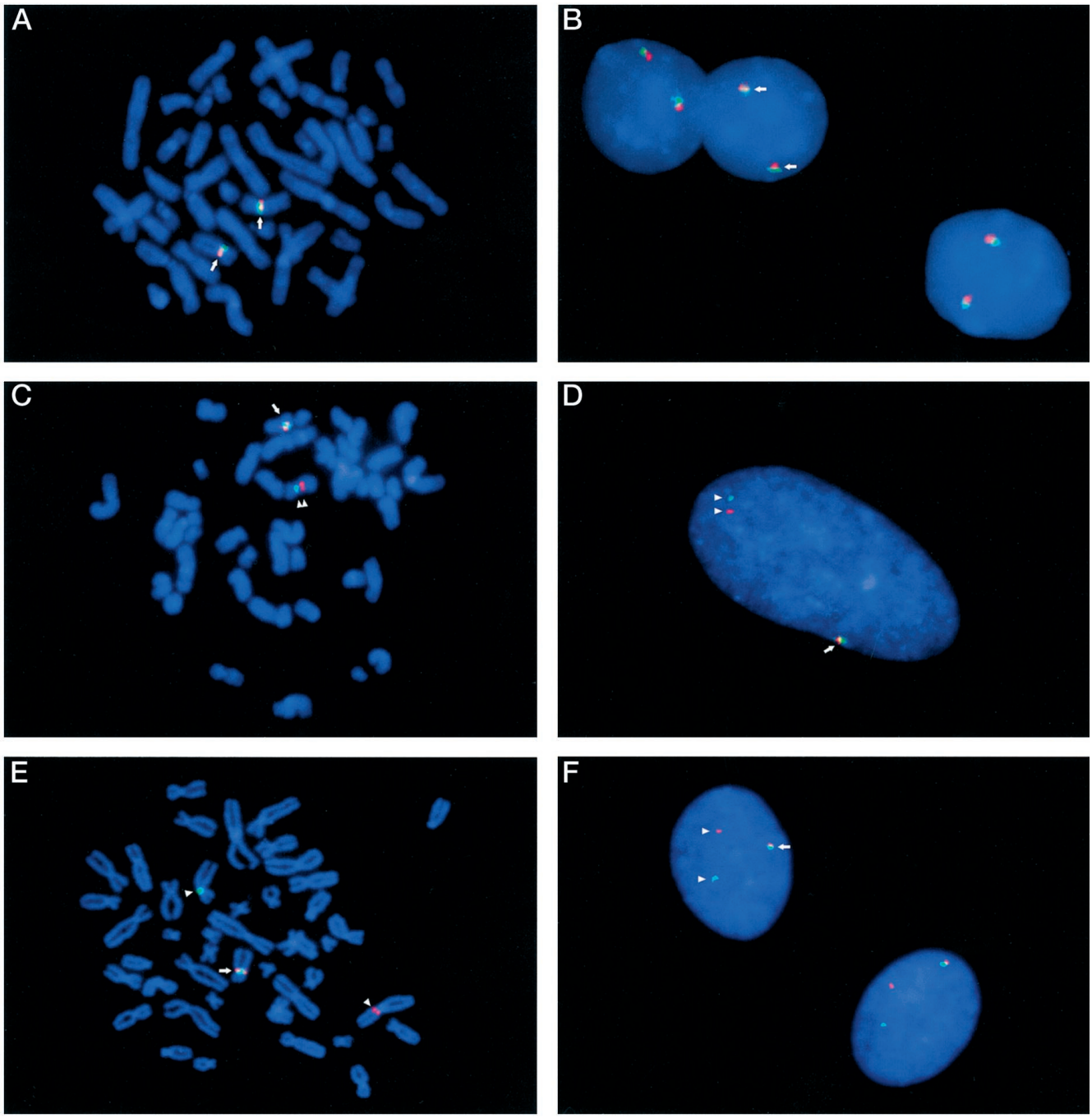

Fig. 1. FISH with *RET*-specific YAC DNA probes. Normal thyroid cells with unrearranged *RET* locus in metaphase spreads (*A*) and interphase nuclei (*B*) showing tightly colocalized red and green signals. Metaphase (*C*) and interphase (*D*) cells from a papillary thyroid carcinoma with a previously proven PTC1 interchromosomal rearrangement showing colocalized red and green signals on normal chromosome 10 and split red and green signals on the affected chromosome 10. Metaphase (*E*) and interphase (*F*) cells from papillary thyroid carcinoma S284 with a balanced translocation affecting the *RET* locus showing colocalized red and green signals on normal chromosome 10 and split red and green signals on two derivative chromosomes der(1)t(1;10) and der(10)t(1;10), respectively. Note the larger physical distance between split signals for the interchromosomal compared with the intrachromosomal rearrangement.

acid 963 of kinectin was fused to amino acid 713 of ret in tumor S271. The COOH-terminal part of ret with the functional TK domain was juxtaposed to the leucine zipper motifs of the NH_2-terminal part of kinectin. Because *KTN1* is expressed in the thyroid and its product can mediate dimerization via coiled-coil domains, we concluded that the 5′ part of *KTN1* fused to the *RET* TK domain is responsible for ectopic expression and ligand-independent activation of the ret TK domain, leading to oncogenic transformation of thyroid cells.

The proposed mechanism of constitutive activation of the ret TK domain in the novel ktn1/ret fusion protein is in accordance with findings demonstrating activation of rearranged ret oncoproteins, *i.e.*, ligand-independent phosphorylation of tyrosine residues, resulting from *RET* rearrangements PTC1–PTC7 as well as from *ELKS/RET*. Similar to these rearrangements, the ret-fused gene *KTN1* is expressed in various tissues (17) and shares with all thus-far-identified ret-fused genes (*H4*, *RIa*, *ELE*, *RFG5*, *HTIF*, *RFG7*, and *ELKS*) the presence of nucleotide sequences coding for proteins with an extremely high probability of forming coiled-coil domains, thus allowing constitutive dimerization of the ret TK domain.

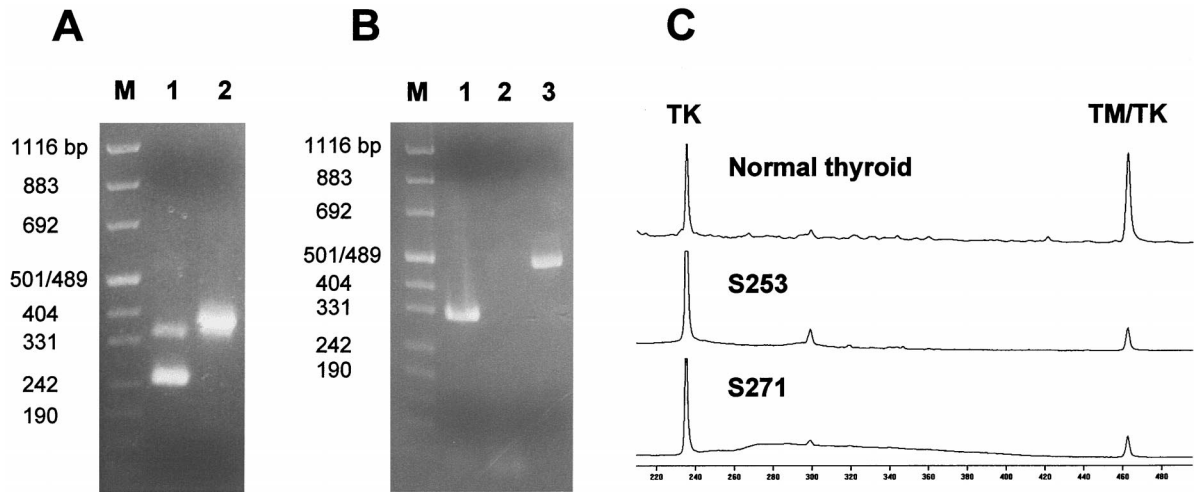

Fig. 2. *A*, identification of *HTIF1/RET* (*Lane 1*) and *RFG7/RET* (*Lane 2*) PCR fragments of the expected size (256/358 bp and 388 bp, respectively) demonstrating the presence of PTC6 and PTC7 rearrangements in tumor samples S299 and S284, respectively. *Lane M*, molecular size standard pUC Mix Marker. *B*, RT-PCR analysis using primers KTN1F and RETSP3 demonstrated the expression of chimeric *KTN1/RET* transcripts (331 bp) in tumor sample S271 (*Lane 1*) but not in the corresponding nontumorous material (*Lane 2*). Expression of the *KTN1* gene in normal human thyroid tissue is confirmed by the amplification of a 494-bp cDNA fragment using KTN1-specific primers (*Lane 3*). *Lane M*, molecular size standard pUC Mix Marker. *C*, semiquantitative RT-PCR and quantification of the relative amounts of the simultaneously generated PCR fragments TM/TK and TK. The clear quantitative shift toward the TK fragment is indicative for the presence of novel *RET* rearrangements in tumors S253 and S271, related to the detected balanced translocations.

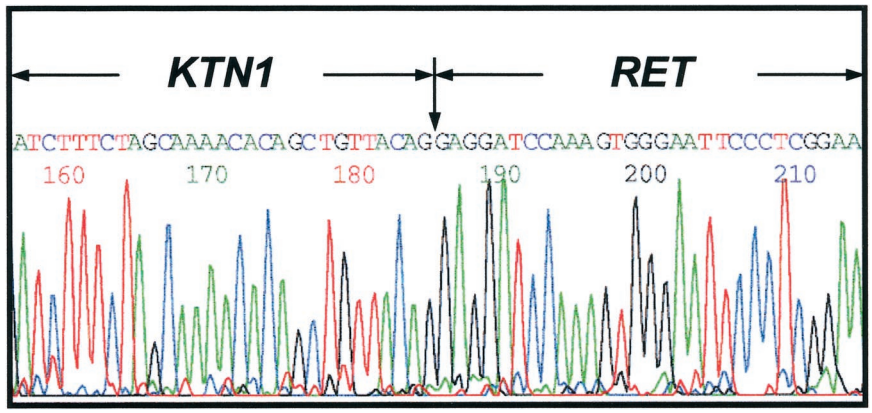

Fig. 3. Sequence analysis of the novel PTC8 rearrangement detected in case S271. *Arrow*, fusion point of the genes *KTN1* and *RET*.

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