**HOOK3-RET**: a novel type of RET/PTC rearrangement in papillary thyroid carcinoma

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**Abstract**

Chromosomal rearrangements of the RET proto-oncogene (RET/PTC) are the common feature of papillary thyroid carcinoma (PTC). In this study, we report the identification, cloning, and functional characterization of a novel type of RET/PTC rearrangement that results from the fusion of the 3'-portion of RET coding for the tyrosine kinase (TK) domain of the receptor to the 5'-portion of the Homo sapiens hook homolog 3 (HOOK3) gene. The novel fusion was identified in a case of PTC that revealed a gene expression signature characteristic of RET/PTC on DNA microarray analysis, but was negative for the most common types of RET rearrangement. A fusion product between exon 11 of HOOK3 and exon 12 of RET gene was identified by 5'RACE, and the presence of chimeric HOOK3-RET protein of 88 kDa was detected by western blot analysis with an anti-RET antibody. The protein is predicted to contain a portion of the coiled-coil domains of HOOK3 and the intact TK domain of RET. Expression of the HOOK3-RET cDNA in NIH3T3 cells resulted in the formation of transformed foci and in tumor formation after injection into nude mice, confirming the oncogenic nature of HOOK3-RET.

**Introduction**

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer and accounts for ~80% of all thyroid malignancies (Hundahl et al. 2000). Genetic alterations along the RET/RAS/BRAF/MAPK signaling pathway have been demonstrated to be crucial in the pathogenesis of papillary carcinomas, as they are found in more than 70% of all tumors and rarely overlap in the same tumor, suggesting that activation of a single effector of this pathway is sufficient for cell transformation (Kimura et al. 2003, Soares et al. 2003, Frattini et al. 2004). The most common genetic alteration of this pathway is the point mutation V600E of the BRAF gene, which is found in ~40% of papillary carcinomas (reviewed in Ciampi & Nikiforov 2005, Xing 2005). Recently, BRAF has also been shown to be activated in papillary carcinomas by chromosomal rearrangement resulting in the AKAP9-BRAF fusion (Ciampi et al. 2005). It is a rare event in sporadic papillary carcinomas and more common in radiation-induced tumors. Approximately, 10% of papillary carcinomas harbor point mutations in one of the RAS genes (N-RAS, H-RAS, K-RAS), with most of these tumors being the follicular variants of papillary carcinoma (De Micco 2003, Zhu et al. 2003). Chromosomal rearrangements involving the RET proto-oncogene is another common alteration in PTC (Fusco et al. 1987, Grieco et al. 1990).

The RET proto-oncogene resides on chromosome 10q11.2 and codes for a membrane receptor tyrosine kinase (TK; Takahashi et al. 1985, Takahashi 1988). It contains three functional domains: an extracellular...
ligand-binding domain with four cadherin-like repeats and one cysteine-rich region, a hydrophobic transmembrane domain, and an intracellular domain containing the TK domain. The ligands of the RET receptor belongs to the group of the glial cell line-derived neutrophic factor family and include neurturin (NRTN), persephin (PSPN), and artemin (ARTN; Airaksinen et al. 1999). Binding of the ligand causes receptor dimerization, autophosphorylation of critical tyrosine residues within the intracellular domain, and activation of the signaling cascade.

In the thyroid gland, RET is expressed at high level in parafollicular C-cells, where point mutations of RET are responsible for the development of medullary thyroid carcinoma. In follicular thyroid cells, RET can be activated by chromosomal rearrangement resulting in the fusion of the 3'-portion of the RET gene to the 5'-portion of several unrelated genes, which is known as RET/PTC rearrangement (Fusco et al. 1987, Grieco et al. 1990). Three types of RET/PTC were originally identified and remain by far the most common in papillary carcinomas. Of them, RET/PTC1 is formed by fusion with the H4 (also known as D10S170, CCDC6) gene (Grieco et al. 1990) and RET/PTC3 by fusion with the NCOA4 (RFG, ELE1) gene (Bongarzone et al. 1994, Santoro et al. 1994). RET/PTC1 and RET/PTC3 are intrachromosomal paracentric inversions since both genes participating in the rearrangement are located on chromosome 10q (Pierotti et al. 1992, Minoletti et al. 1994). In contrast, RET/PTC2 is a translocation between chromosomes 10 and 17, resulting in the RET fusion with the regulatory subunit R1z of the cAMP-dependent protein kinase A (Bongarzone et al. 1993). More recently, several novel types of RET/PTC have been described in single cases of PTC, all of which are interchromosomal translocations (Klugbauer et al. 1998, 2000, Klugbauer & Rabes 1999, Nakata et al. 1999, Corvi et al. 2000, Salassidis et al. 2000, Saenko et al. 2003).

In a previous study of PTC using DNA microarray analysis, we determined a distinct expression profile for the RET/PTC rearrangement and established a highly accurate mutational classifier based on the expression of several genes (Giordano et al. 2005). We identified a case of papillary carcinoma in which the classifier predicted the presence of RET/PTC rearrangement, despite the negative testing of the tumor RNA for RET/PTC1 and RET/PTC3 rearrangements by RT-PCR. By fluorescence in situ hybridization (FISH), we demonstrated that the tumor cells indeed harbor a RET rearrangement, but one that did not correspond to the most common RET/PTC1 or RET/PTC3 types. In this study, we performed further analysis of this case which resulted in the identification, cloning, and functional characterization of a novel type of RET/PTC rearrangement.

**Materials and methods**

**Tumor samples and cell lines**

Snap frozen tissue from the index case was obtained from the University of Michigan Health System through the Tissue Procurement Service with Institutional Review Board (IRB) approval. The index case was a 14-year-old female with a 7×4×4 cm papillary carcinoma of the right lobe. The tumor morphology was classical papillary type and there were four positive central compartment lymph nodes. There was no history of prior radiation exposure. Frozen tissue samples from 129 PTC were obtained from the Department of Pathology, University of Cincinnati following a protocol approved by the University of Cincinnati IRB and through the Cooperative Human Tissue Network.

**Fluorescence in situ hybridization (FISH)**

FISH was performed as previously described (Ciampi et al. 2005). Briefly, a mix of the two bacterial artificial chromosome (BAC) clones RP11-168L22 (GenBank accession no. AC068707) and RP13-368N15 (GenBank accession no. AL591169) were used to design a probe spanning the RET gene. The clone RP11-122A17 (GenBank accession no. AC110275) was used as a probe for the HOOK3 gene. The clones were purchased from BAC/PAC Resources, Children’s Hospital, Oakland. The extracted DNA was labeled with Spectrum-Green-dUTP and SpectrumRed-dUTP using a nick translation kit (Vysis Inc., Downers Grove, IL, USA). Touch-preparations from the tumor were prepared from snap frozen tissue and fixed in 3:1 methanol:acetic acid, pretreated with 10 mg collagenase H (Sigma), and co-denatured with 10 ng each labeled probe. Hybridization was carried at 37 °C overnight. Microscopy was performed with a Leica Microsystem TCS 4D confocal fluorescence microscope with digital image capture (Leica, Wetzlar, Germany).

**5’RACE**

Total RNA from the index case was extracted using Trizol Reagent (Invitrogen) and subjected to 5’RACE using the 5’RACE System for Amplification of cDNA Ends (Invitrogen). cDNA synthesis was performed using a previously published primer corresponding to exon 13 of the RET gene, 5’-CTGCTTCAGGACGTTGAA-3’.
(Santoro et al. 1994). Subsequent PCRs utilized primer 5'-GCAGGTCTCCGCAGCTCACTC-3' for the first reaction and 5'-CTTTCAGCATCTCTCAAGG-3' (Santoro et al. 1994) for the second round of amplification. The RACE products were excised from the gel, purified and sequenced using an automated ABI 377 Sequencer (Perkin–Elmer, Waltham, MA, USA).

**Cloning and sequencing analysis**

The open reading frame (ORF) of the chimeric cDNA was amplified using primers spanning the start codon of HOOK3 (5'-CACCATGGTTCAGCGTAGAG-3') and the stop codon of RET (5'-ACTATCAAACGTGTCATTAATTT-3'). The PCR product was electrophoresed on agarose gel, purified, and cloned into the pcDNA3.1D/V5-His-TOPO expression vector using the pcDNA3.1 Directional TOPO Expression kit (Invitrogen). Several clones were fully sequenced and the clone I-9 was chosen for further experiments.

**RT-PCR experiments**

Total RNA was extracted using Trizol Reagent (Invitrogen), reverse transcribed, and amplified by PCR using primers flanking the HOOK3-RET fusion point: 5'-AGGCGGCGTAAACTCTT-3' located in the exon 11 of HOOK3 and 5'-TCCAAATTCCTCTCCTA-3' in the exon 12 of RET, with an expected product of 195 bp. Amplification was carried out for 35 cycles (94 °C for 40 s, 55 °C for 1 min, and 72 °C for 1 min 30 s). In order to study the expression of the wild-type HOOK3, semi-quantitative RT-PCR was performed for 30 cycles using primers 5'-TCCAAATTCCTCTCCTA-3' and 5'-TGTTCATGCCTGTCTTTTTT-3', with the expected amplification product of 246 bp. The results were normalized using amplification of the housekeeping gene phosphoglycerate kinase (PGK), as described elsewhere (Argani et al. 1998).

**Western blot analysis**

Western blotting was performed as previously described (Kunzli et al. 2002). Twenty microgram of proteins were loaded on a 7.5% polyacrylamide gel and subjected to SDS–PAGE. After the electro-transfer, membranes were incubated with anti-RET mouse monoclonal antibody (clone RET-X-D210) generated against the C-terminus of the RET protein in our laboratory and previously validated (Knauf et al. 2003). The dilution of the antibody was 1:250. HRP-goat-antimouse secondary antibody (Zymed, San Francisco, CA, USA) was used for the detection. Protein extracts from liver, medullary thyroid carcinoma, and TPC1 cell line harboring RET/PTC1 rearrangement (Ishizaka et al. 1990) were used as controls.

**Transformation assays**

NIH 3T3 cells were plated in six-well plates at a density of 3.4×10^5 and transfected 24 h later using Lipofectamine (Invitrogen) with 16 μg pcDNA3.1D HOOK3-RET plasmid. Cells transfected with the empty vector were used as a negative control. After 1 day, cells were split into three 100 mm^2 dishes and cultured in DMEM with 10% fetal bovine serum (FBS). For the focus assay, cells were cultured for 21 days, stained with 0.5% crystal violet, and foci counted. For stable cell lines, cells were mass selected for 21 days in the media supplemented with 350 μg/ml G-418 Sulfate (Invitrogen). A total of 5×10^6 pooled cells were suspended in 0.4 ml culture media and then injected subcutaneously into the flank of homozygous Nu/Nu female mice (Charles River Laboratories, Wilmington, MA, USA). Cells expressing HOOK3-RET were injected into the left flank and cells containing empty vector were injected into the right flank of each of the six mice. Tumors were removed 20–26 days later, measured, weighed, and fixed in 10% formalin for histologic evaluation.

**Results**

We studied a case of papillary carcinoma that had revealed an expression profile consistent with RET/PTC rearrangement in our previous study (Giordano et al. 2005). The presence of RET rearrangement in the majority of tumor cells was confirmed with FISH using a probe spanning the RET gene. A split of one of the two RET signals was noted in the majority of tumor cells (Fig. 1A).

In order to identify the partner gene of RET in this rearrangement, we performed the 5'RACE assay using total RNA from the index case and specific primers for the RET gene designed along its 3'portion. Several bands were present after the nested amplification and a prominent band was gel purified and sequenced. The sequencing revealed that exon 12 of RET was fused in-frame with exon 11 of the Homo sapiens hook homolog 3 (HOOK3; GenBank accession number: BC056146; Fig. 2A). The breakpoint within the RET gene was in the same position as in all the other known types of RET/PTC rearrangement. The novel fusion ORF resulted in a transcript with a predicted size of 2331 bp (1122 bp from HOOK3 and 1209 bp from RET) and a protein of 776 amino acids with a predicted
molecular weight of 88.2 kDa (Fig. 2B). The presence of the fusion between these two genes was confirmed at a genomic level by two-color FISH using probes corresponding to the RET and HOOK3 genes (Fig. 1B).

Since the pattern of the HOOK3 gene expression in human thyroid cells was not well studied, we analyzed it by semi-quantitative RT-PCR in normal thyroid (NT) tissue and in several other tissues, including liver, skeletal muscle, and kidney. Abundant HOOK3-RET mRNA was found in all NT tissue samples and in other tissues studied (Fig. 3).

To confirm the presence of the fusion transcript in the index case, we performed RT-PCR with primers flanking the identified breakpoint of HOOK3-RET. The expected 195 bp product was successfully amplified (data not shown). Western blot analysis using an

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**Figure 1** Fluorescence in situ hybridization (FISH) on interphase tumor cell nuclei from the index case. (A) Hybridization with the RET probe only shows split of one of the signals (arrows) indicating the presence of the RET gene rearrangement. (B) Two-color FISH with the RET probe (green) and the HOOK3 probe (red) confirming the HOOK-RET fusion (arrow). Full color version available at http://erc.endocrinology-journals.org.

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**Figure 2** (A) Sequence of the 5' RACE product showing the fusion between HOOK3 and RET. (B) Genomic organization of the HOOK3 and RET genes and the HOOK3-RET fusion. The boxes indicate exons and the lines indicate introns. Black arrows indicate the location of breakpoint in the two genes. The chimeric cDNA is shown at the bottom of the panel with indication of its size (bp) and the number of corresponding amino acids.
antibody against the C-terminal portion of RET revealed a band of \( \sim 88 \) kDa in the index case which corresponds to the predicted size of the chimeric HOOK3-RET protein (Fig. 4).

To test the oncogenic characteristics of the novel fusion gene, the full-size coding sequence of HOOK3-RET cDNA was amplified by RT-PCR and cloned into the pcDNA3.1D/V5-His-TOPO mammalian expression vector. Several clones were screened by restriction analysis and sequenced. The clone I-9 was chosen for the functional studies (GenBank accession no. NM_032410).

The ability of HOOK3-RET protein to induce cell transformation was studied in an NIH3T3 cells focus assay. Cells transfected with the HOOK3-RET ORF cDNA formed transfected foci at high efficiency when compared with cells transfected with empty vector (Fig. 5A). In addition, NIH3T3 cells stably transfected with HOOK3-RET formed tumors in nude mice as early as 10 days after injection. Tumors formed in all mice injected and grew to 0.78–1.22 g (20–26 mm in size) 3 weeks after injection. In contrast, no tumors were detected after injection of cells transfected with empty vector (Fig. 5B). These results demonstrate that HOOK3-RET functions as an oncogene and is able to transform NIH3T3 cells both in vitro and in vivo.

The prevalence of the HOOK3-RET fusion was studied by RT-PCR with primers flanking the fusion point in a series of 129 consecutive PTC cases. No additional tumors were positive for the fusion indicating that this novel RET/PTC rearrangement is not a common event in thyroid papillary carcinomas from the general population.

**Discussion**

In this study, we report the identification of a novel RET/PTC rearrangement that results from fusion of the RET and HOOK3 genes. Since the identification of the first RET partner in a chromosomal rearrangement reported in 1990 (Grieco et al. 1990), 12 different types...
of RET/PTC have been identified resulting from the fusion of RET to different partner genes (Table 1; Bongarzone et al. 1993, 1994, Santoro et al. 1994, Klugbauer et al. 1998, 2000, Klugbauer & Rabes 1999, Nakata et al. 1999, Corvi et al. 2000, Salassidis et al. 2000, Saenko et al. 2003). RET/PTC1 and RET/PTC3 result from intrachromosomal inversions, and constitute the two most common RET/PTC rearrangement types found in sporadic and radiation-associated papillary carcinomas. RET/PTC2 and the more recently identified rare types of RET/PTC are all interchromosomal translocations. Most of these rare RET/PTC types have been found in papillary carcinomas from patients with a history of either environmental (after Chernobyl) or therapeutic exposure to ionizing radiation (Klugbauer et al. 1998, 2000, Klugbauer & Rabes 1999, Corvi et al. 2000, Salassidis et al. 2000, Saenko et al. 2003). The one exception is the ELKS-RET fusion identified in a case of papillary carcinoma with no apparent history of radiation exposure (Nakata et al. 1999). In the present study, we identify yet another novel RET/PTC rearrangement in a patient with no radiation exposure history.

The novel rearrangement type reported herein resulted from the fusion of RET to yet another gene, HOOK3, located on chromosome 8. The presence of RET/PTC was predicted in this tumor based on the analysis of gene expression profiles obtained by DNA microarray analysis (Giordano et al. 2005). The analysis defined distinct gene expression signatures for papillary carcinomas carrying specific types of mutations, i.e., BRAF, RAS, and RET/PTC. One papillary carcinoma showed an expression profile highly consistent with the presence of RET/PTC, but revealed no RET/PTC1 or RET/PTC3 rearrangement, suggesting that it may harbor either a known rare type of rearrangement or a novel type of RET/PTC. Further analysis now reveals a novel RET/PTC rearrangement in this tumor, HOOK3-RET. To our knowledge, this is only the second example of a novel chromosomal rearrangement being detected based on the information obtained by gene expression profile analysis. Previously, the analysis of gene expression profiles allowed identification of the fusion between the TMPRSS2 and either the ETV1 or the ERG genes in prostate cancer (Tomlins et al. 2005). The identification of HOOK3-RET provides further confirmation of the accuracy of the identified transcription signatures associated with specific mutations. It also offers evidence that different types of RET/PTC rearrangement lead to similar effects with respect to alteration of the expression of many genes. In addition, it validates the use of such an approach to search for novel genetic alterations in papillary carcinomas that show transcription signatures of other mutations, such as those of RAS genes, but lacking mutations affecting the known hot spots within these genes (Giordano et al. 2005).

It has been established that different RET partner genes involved in RET/PTC rearrangement share several common characteristics important for the oncogenic function of the chimeric gene. They are all expressed in thyroid follicular cells, providing an active promoter for the fusion gene. In addition, they all encode putative dimerization domains, either a coiled–coil or leucine zipper, essential for dimerization and ligand-independent activation of the RET (TK; Tong et al. 1997, Jhiang 2000). As for the RET gene, virtually all breakpoints occur within its 1.8 kb intron 11, leaving intact the TK domain of the receptor and therefore enabling the RET/PTC oncoprotein to bind SHC via Y1062 and activate the RAS–RAF–MAPK pathway in thyroid cells (Knauf et al. 2003).

All of these structural features can be found in the HOOK3-RET chimeric gene. HOOK3 encodes a protein belonging to a recently identified family of human cytosolic coiled-coil proteins that link...
cytoplasmic organelles to microtubules (Walenta et al. 2001). The three known human HOOK proteins, HOOK1, HOOK2, and HOOK3, contain conserved N-terminal domains which attach to microtubules, and more variable C-terminal domains which mediate binding to different organelles. The C-terminal portion of HOOK3 binds to Golgi membranes in vitro, suggesting that it may participate in defining the organization and localization of the mammalian Golgi complex within the cell (Walenta et al. 2001). Similar to all other family members, the HOOK3 protein contains an extended central coiled-coil motif, which was shown to mediate homodimerization in the Drosophila Hook protein (Kramer & Phistry 1996, Sevrioukov et al. 1999). In the HOOK3-RET fusion, the first 11 exons of the HOOK3 gene are fused to exons 12–20 of the RET gene. Two large coiled-coil domains of HOOK3 encoded by exons 7–11 (aa. 171–226 and 244–359) are preserved in the HOOK3-RET protein, indicating that it possesses a dimerization domain found in all known RET fusion partners. In the RET gene, the breakpoint is located within intron 11, similar to most other rearrangement types. Finally, we confirmed that the wild-type HOOK3 gene is expressed in thyroid follicular cells, providing an active promoter to drive the expression of the TK domain of RET. These qualities are expected to enable the HOOK3-RET with oncogenic properties, which indeed were identified in both in vitro and in vivo experiments.

In summary, we report a novel RET/PTC rearrangement resulting from RET fusion to the HOOK3 gene which leads to the formation of an oncogene.

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References

De Micco C 2003 Ras mutations in follicular variant of papillary thyroid carcinoma. American Journal of Clinical Pathology 120 803–804.


Klugbauer S & Rabes HM 1999 The transcription coactivator HATF1 and a related protein are fused to the RET receptor tyrosine kinase in childhood papillary thyroid carcinomas. *Oncogene* **18** 4388–4393.


Salassidis K, Bruch J, Zitzelsberger H, Lengfelder E, Kellerer AM & Bauchinger M 2000 Translocation t(10;14)(q11.2;q22.1) fusing the kinetin to the RET gene creates a novel rearranged form (PTC8) of the RET proto-oncogene in radiation-induced childhood papillary thyroid carcinoma. *Cancer Research* **60** 2786–2789.


