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Rearrangement Analysis in Archival Thyroid Tissues: Punching Microdissection and Artificial RET/PTC 1–12 Transcripts

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Background. **In few papillary thyroid carcinomas (PTC) and oxyphilic thyroid carcinoma, the clinical impact of the 15 known *RET* hybrid oncogene variants (RET/PTC 1 to 12, 1L, 3r2, 3r3) is subject to controversial discussions. Large patient cohorts and exploitation of pathological thyroid tissue archives are essential to study the prognostic significance of RET/PTC chimeras.**

Materials and Methods. **Formalin-fixed and paraffin-embedded thyroid neoplasms were subjected to manual punching macrodissection and subsequent extraction of total RNA. Following reverse transcriptase polymerase chain reaction (RT-PCR)-based screening for *RET* rearrangements, hybrid-specific expression analyses were carried out for samples indicative of chimeric transcripts. Due to lack of tissue specimen harboring the rare *RET* chimeras, artificially constructed hybrid sequences of all known RET/PTC variants served as PCR controls.**

Results. **Manual punching dissection successfully diminished *RET* wild-type contamination originating from C-cells dispersed throughout normal thyroid tissues. The average amount of 27.4 μg RNA extracted allowed for repeated molecular analyses (>60 PCRs). Hybrid-specific expression analysis identified 10 of 15 *RET* rearrangements (8x RET/PTC 1, 2x RET/PTC 3, 5x RET/PTC x) to be found in 54 oxyphilic thyroid tumors examined. Successful amplification of each artificial hybrid sequence ensured the absence of rare chimeric transcripts. Therefore, RET/PTC x represent either common chimeras not amplifiable due to archival RNA degradation or truly novel hybrid oncoproducts.**

Conclusions. **The fast and simple techniques described here were used to examine oxyphilic carcinomas and adenomas. These microdissection and RT-PCR procedures can easily be put into practice in any molecular biology research laboratory to enable screening of large numbers of archival thyroid tumors for known as well as yet unknown *RET* rearrangements.**

Key Words: **RET/PTC; rearrangement; oxyphilic thyroid tumors; archival tissue; artificial transcript; microdissection.**

INTRODUCTION

The RET proto-oncogene (rearranged during transfection) denotes a transmembrane receptor tyrosine kinase mediating signal transduction by transphosphorylation of tyrosine residues. The RET protein forms receptor complexes with members of the coreceptor family GDNF α (GFR α 1–4). These coreceptors determine the specificity to the ligand family of neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN), and persephin (PSPN) [1]. While in the adult thyroid gland neural crest-derived cells—parafollicular C-cells and their malignant counterparts, medullary thyroid carcinomas (MTCs)—express the wild-type tyrosine kinase receptor, RET was found to be lacking in the follicular cell-derived thyrocytes after embryonic development. Interestingly, the wild-type variant was demonstrated in lymph node metastases of few papillary thyroid carcinomas (PTCs) only, implying that a minority of these cancers regain RET expression by yet unknown molecular alterations [2].

In benign and malignant thyroid tumors arising from follicular cells, the *RET* gene is frequently a target of oncogenic activation through balanced chromosomal rearrangements. These rearrangements fuse the

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tyrosine kinase (TK) domain of the RET receptor to promoter regions of different, ubiquitously expressed genes. In somatic cells, these rearrangements lead to constitutive ectopic expression of the resulting chimeric oncogenes which, in turn, induces increased tyrosine kinase activity [1, 3]. Due to the fact that wild-type RET is not active in adult normal thyroid tissue, the reciprocal transcripts (i.e., the chimeras harboring the 3′ extracellular RET domain) are rarely expressed [4–6].

To date, 11 different fusion partner genes have been reported forming, due to variable breakpoints, at least 15 different *RET* hybrid oncogenes, designated RET/PTC 1 to 12, RET/PTC 1 long, and RET/PTC Δ3 (3r2, 3r3) (Fig. 1), [1, 7]. *RET* rearrangements have been described in benign thyroid lesions, such as adenomas and thyroiditis, in benign and malignant oxyphilic tumors of the thyroid, and especially in PTC. Hybrid *RET* oncogenes are even considered a hallmark of the papillary variant of thyroid cancers. In contrast, *RET* chimeras were not detected in follicular thyroid carcinomas (FTCs), except for sporadic reports describing the expression of RET/PTC 1 in FTCs with a history of low-dose external-beam irradiation [8] or in Japanese FTCs [9]. The differentiation of FTCs and follicular variants of PTCs lacking any papillary architecture requires histopathological and cytopathological expertise.

Although it is generally agreed that RET/PTC rearrangements take place in early thyroid carcinogenesis, a potential genotype-phenotype correlation (the prognostic significance of RET oncoproteins [2, 7, 10–13] as well as their role as targets for cancer treatment strategies [1]), is subject to ongoing controversial discussion. To elucidate the clinical characteristics and prognosis of thyroid tumors harboring *RET* gene rearrangements, further molecular studies on large numbers of respective patients are required with regular follow-ups over several decades. This task is hampered by the rarity of panels of fresh-frozen tissue samples available for molecular analysis, whereas pathological archives represent the ideal source of tissues.

Therefore, we aimed to devise an improved analytical assay for identification of *RET* rearrangements in formalin-fixed, paraffin-embedded tumor tissues. When using a PCR-based technique for RET/PTC expression analysis in archival pathological tissues, five problems have to be addressed. First, total RNA extracted from archival tissues usually consists of short RNA fragments of not more than approximately 200 base pairs [14], necessitating appropriate PCR design. Second, repeated PCR analysis requires a RNA extraction method yielding high amounts of total RNA at low expense, thus necessitating few working hours only. Third, the wild-type RET receptor is expressed in parafollicular C-cells, which are dispersed throughout the thyroid gland. This wild-type RET mRNA expression affects PCR analysis for RET/PTC hybrids and should, therefore, be minimized by microdissection of the tumor and removal of C-cell-containing normal thyroid tissue. Fourth, a comprehensive search for *RET* rearrangements should, ideally, not only include all 15 known hybrid oncoproducts, but additionally allow for the detection of yet unknown RET/PTC chimeras. Fifth, due to the fact that detection of the very rare variants RET/PTC 5-12 is only described in singular tumors scattered worldwide, there is a lack of appropriate control samples required for *lege artis* PCR analysis.

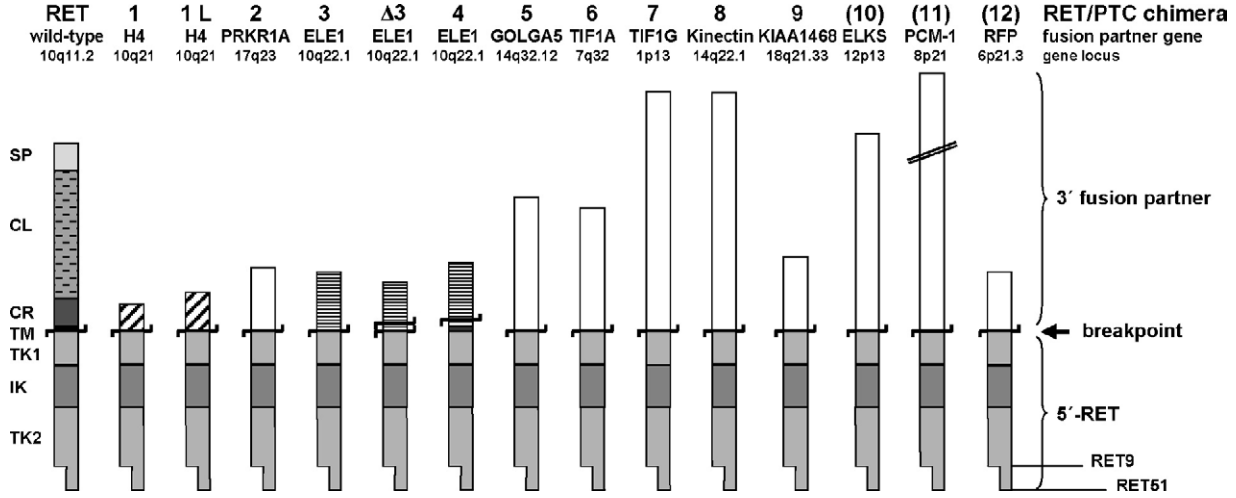

FIG. 1. Up to date, 15 different RET hybrid oncoproducts, designated RET/PTC 1, 1 long, 2, 3, Δ3, 4 to 12, are reported. The chimeras are formed by fusing *RET* proto-oncogene sequences, coding for the tyrosine kinase (TK) domains, to amino-terminal sequences of 11 different fusion partner genes, which results in activation of the chimeric gene and therefore to subsequent nonphysiological expression of the RET protein TK domain. Variant intronic breakpoints are found within *RET* (RET/PTC 4) as well as in the fusion partner genes H4 (RET/PTC 1L) and ELE1 (Δ3 = 3r2 and 3r3), respectively. Both alternatively spliced RET variants are depicted (RET9, RET51). RET domains: SP = signal peptide; CL = cadherin-like; CR = cysteine-rich; TM = transmembrane, TK1 and 2 = split tyrosine kinase; IK = interkinase.