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Molecular profile of hyalinizing trabecular tumours of the thyroid: High prevalence of *RET/PTC* rearrangements and absence of *B-raf* and *N-ras* point mutations☆

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Abstract

Hyalinizing trabecular tumour (HTT) of the thyroid is a neoplasm of follicular derivation that shares several morphological similarities with papillary thyroid carcinoma (PTC). In this study, we investigated the prevalence of *B-raf* point mutations, *RET/PTC* rearrangements and *N-ras* point mutations in a large HTT series (28 samples). Twenty benign thyroid lesions and 10 PTC served as control cases. A high (47%) prevalence of *RET/PTC* rearrangements was found in HTT. By contrast, neither *B-raf* nor *N-ras* mutations were found in HTT. These findings suggest that, although *RET/PTC*, *N-ras*, and *B-raf* proteins may act along the same signalling cascade, the biological and morphological outcome of their oncogenic activation is not completely overlapping. Thus, in clinical practice, the detection of *B-raf* mutations in a thyroid follicular tumour may prove to be a valuable tool, supplementing histological examination, and allowing a differential diagnosis between PTC and HTT.

Keywords: Thyroid; Oncogene; Kinase; *RAF*; *RET*; Papillary carcinoma

1. Introduction

In 1987, Carney and co-workers described a previously unrecognised thyroid tumour with unusual but benign features and named it "hyalinizing trabecular adenoma". The tumour was characterised by circumscription or encapsulation, trabecular growth pattern, polygonal and elongated cells, nuclear cytoplasmic inclusions and grooves, hyaline material, dilated sinusoids, laminated calcospherites, and cytoplasmic yellow bodies [1,2]. The peculiar nuclear features of the tumour have raised intriguing questions regarding its morphological and biological classification. Although occasional aggressive hyalinizing trabecular neoplasms with vascular invasion have been described [3], the tumour as described by Carney colleagues [1] is generally accepted as being benign. The non-committal title "hyalinizing trabecular tumour" (HTT) is currently favoured

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for the lesion. Phenotypic heterogeneity on morphological and immunochemical grounds is very common in HTT [4,5]. The neoplasm shares histological and cytological features with papillary thyroid carcinoma (PTC). These similarities include enlarged and clear nuclei with inclusions and grooves and calcifications resembling psammoma bodies [6]. A relationship between the neoplasms is further supported by the frequent concurrence of PTC and HTT [6]. In addition, tumours show positivity for epithelial-type cytokeratins (especially CK19) [7]. However, HTT is largely negative or only weakly positive for galectin-3, while most PTC show strong staining, suggesting a possible difference between the two tumours [8].

Molecular genetics advances over the past two decades offer unique opportunities today for studying the relationships between morphology, biology and genetics of thyroid tumours. Previous investigations have shown that gene rearrangements of *RET* and point mutations in *B-raf* oncogenes are hallmarks of PTC. *RET* encodes the tyrosine kinase (TK) membrane receptor for glial cell line-derived neurotrophic factors [9]. In PTC (2.5–40% of the cases), chromosomal inversions or translocations at 10q11.2 lead to the fusion of the *RET* TK-domain to heterologous genes (*RET*/*PTC* oncogenes) and consequently to the activation of its signalling and transforming properties [10]. *RET*/*PTC1* (*H4-RET*) and *RET*/*PTC3* (*RFG-RET*) are the most prevalent variants [11]. *RET*/*PTC* rearrangements have also been found in clinically occult papillary microcarcinomas [12], and Hurthle cell tumours [13]. Activating point mutations in *B-raf* were identified as another common feature of PTC (approximately 45% of the cases) [14]. All the *B-raf* mutations identified thus far affected nucleotide 1796 in exon 15, resulting in a thymine-to-adenine transversion, which translates into valine to glutamate substitution at residue 599 (V599E) [14]. *B-raf* belongs to the *RAF* family of serine/threonine kinases and it is located downstream of *RAS* small GTPases and upstream of *MEK* in the classic *MAPK* cascade [15]. This pathway is activated by all growth factor receptors with tyrosine kinase activity, and thus, presumably, by *RET*/*PTC* as well [16]. More rarely, PTCs, especially those belonging to the follicular variant, feature activating point mutations in *RAS* family members, with the *N-ras* mutation at codon 61 being the predominant one [17,18].

Papotti colleagues [19] and Cheung and colleagues [20] reported that HTT frequently (4 out of 14 and 6 out of 8 cases, respectively) featured *RET*/*PTC* rearrangements. More recently, Trovisco and co-authors reported that none out of 5 HTT cases showed *B-raf* mutations [21]. Analysis of *Ras* mutations in HTT samples have never been reported. Here, we sought to perform a comprehensive molecular analysis of a large HTT series.

2. Materials and methods

2.1. Tumours

Archival paraffin-embedded thyroid samples from 58 patients were retrieved from the files of Pathology Departments of the Mayo Clinic (Rochester, MN) and Hopital de L'Antiquaille (Lyon, France) following informed consent. The sample series included 28 HTT. As controls, we analysed eight non-toxic multinodular goitres (MNG), 12 follicular adenomas (FA) and 10 papillary carcinomas (PTC), including eight classic, one tall-cell and one follicular variant tumours. Clinico-pathological data including gender, age, size of tumour, location, associated thyroid lesions and metastatic deposits were collected (Table 1). Resected tumours were fixed in 10% neutral-buffered formalin and embedded in paraffin blocks. Sections (4 μM thick) were stained with haematoxylin and eosin for histopathological interpretation. The diagnosis of HTT was made in accordance with established criteria [22]. Additional sections (20 μM thick) were cut from the paraffin blocks, immersed in xylene for 20 min to remove the

Table 1
Patients, tumours and prevalence of genetic alterations in selected thyroid lesions

Tissue type	Age (in years) (average)	Female/male ratio	Tumour size (average in cm)	*PTC1*	*PTC3*	*B-raf* [e]	*N-ras*
HTT[a]	44 ± 10	4:1	2.1 ± 0.9	10/28 (36%)	3/28 (11%)	0/28	0/28
FA[b]	40.6 ± 12.3	5:1	3 ± 1.7	0/12	0/12	0/12	1/10 (10%)
MNG[c]	47.6 ± 13	7:1		0/8	0/8	0/8	0/8
PTC[d]	45.7 ± 11	4:1	3.8 ± 1.8	1/10 (10%)	2/10 (20%)	4/10 (40%)	0/10

± Standard error of the mean.
[a] Hyalinizing trabecular tumour.
[b] Follicular adenoma.
[c] Multinodular goitre.
[d] Papillary thyroid carcinoma.
[e] Identical results were obtained by the sequencing of polymerase chain reaction (PCR) products amplified either from DNA or RNA.

paraffin, washed in ethanol, and processed for nucleic acid extractions.

2.2. Detection of B-raf point-mutations

B-raf point mutations were analysed at both the DNA and RNA level in all of the samples. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK). DNA from each tumour sample was resuspended in 200 μl H_2O; 5 μl were used as a template for the polymerase chain reaction (PCR) amplification. RNA was extracted using the RNeasy Mini Kit (Qiagen); 500 ng of RNA were used for reverse transcription (RT). The quality of the RNA was checked by amplifying a 111 bp fragment of the β-actin housekeeping gene. The sequences of the forward and reverse β-actin primers were as follows:

forward, 5′-TCG TGC GTG ACA TTA AGG AG-3′;
reverse, 5′-GTCAGGCAGCTCGTACT-3′.

RNA from all the samples resulted in sufficiently good quality for mutational screening.

Exons 11 and 15 of *B-raf* were separately amplified because, although the only *B-raf* mutation detected in PTC so far is the V599E mutation in exon 15, mutations in exon 11 have been found in other tumour types [15]. For genomic DNA, PCR primers were designed to amplify target exons and approximately 50-bp flanking intron sequences in both upstream and downstream directions and were as follows:

Exon 11-forward, 5′-TCC CTC TCA GGC ATA AGG TAA-3′;
Exon 11-reverse, 5′-CGA ACA GTG AAT ATT TCC TTT GAT-3′;
Exon 15-forward, 5′-TCA TAA TGC TTG CTC TGA TAG GA-3′;
Exon 15-reverse, 5′-GGC CAA AAA TTT AAT CAG TGG A-3′.

For RT-PCR, primers were as follows:

Exon 11-forward, 5′-GAC GGG ACT CGA GTG ATG AT-3′;
Exon 11-reverse, 5′-CTG CTG AGG TGT AGG TGC TG-3′;
Exon 15-forward, 5′-GCA CAG GGC ATG GAT TAC TT-3′;
Exon 15-reverse, 5′-GAT GAC TTC TGG TGC CAT CC-3′.

Reactions were performed in a 25 μl mixture containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$ (pH 8.3), 0.2 mM deoxynucleoside triphosphates (dNTP), 8 pmol of primers, and 1 U of AmpliTaq DNA Polymerase (Applied Biosystems, Warrington, UK). Cycling conditions were as follows: initial denaturation (94 °C, 5 min), followed by 40 cycles (denaturation, 94 °C for 30 s; annealing, 60 °C for 30 s; synthesis, 72 °C for 60 s), and by a final extension of 5 min at 72 °C. Amplification products were separated on 2% agarose gel and visualised by ethidium bromide staining. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced (ABI PRISM DNA sequencer, Applied Biosystems) by using the forward primers described above. The thyroid carcinoma cell lines FB1, BHT101 and 8505C were used as positive controls [23]. Cells were grown in Dulbecco Modified Eagle Medium containing 10% fetal bovine serum (GIBCO, Paisley, PA).

2.3. Detection of RET/PTC rearrangements

Roughly 90% of *RET/PTC* rearrangements found in thyroid tumours are *RET/PTC1* or *RET/PTC3* [11]. Therefore, the search was focused on these two variants. *RET/PTC* rearrangements were analysed by RT-PCR in the 58 samples. Forward primers, designed on the coiled-coil domains of the *RET* fusion partners (*H4* for *RET/PTC1* and *RFG* for *RET/PTC3*), were as follows:

RET/PTC1: 5′-ATT GTC ATC TCG CCG TTC-3′;
RET/PTC3: 5′-TGG AGA AGA GAG GCT GTA TC-3′;

Reverse primers were:

RET/PTC1: 5′-TGC TTC AGG ACG TTG AAC-3′;
RET/PTC3: 5′-CGT TGC CTT GAC TTT TC-3′;

Five hundred nanograms of RNA were reverse transcribed and subjected to 40 cycles of PCR (94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min). The product was analysed on a 2% agarose gel and hybridised with a *RET* probe covering the TK domain. The amplified products were also sequenced to confirm the rearrangement. Amplification without previous reverse transcription was performed as a negative control in all the cases. Positive controls were tumours samples harbouring *RET/PTC* rearrangements [23]. Levels of β-actin transcripts were measured by RT-PCR for normalisation.

2.4. Detection of N-ras point mutations

Mutations of *N-ras* were studied at the DNA level in the 58 samples. Point mutations in codon 61 of *N-ras* were detected by LightCycler PCR, as previously described in Ref. [17]. The method is based on rapid-cycle PCR amplification of the locus containing a mutational hot spot on the LightCycler (Roche Molecular Biochemicals, Germany) using a hybridisation probe format, fol-

lowed by fluorescence melting curve analysis (FMCA). This format requires a pair of primers and two fluorescently labelled oligonucleotide probes and relies on fluorescence resonance energy transfer between the two probes that hybridise adjacent to one another. One of the two probes was designed to span the mutation site; the perfectly matched and mis-matched probe–target duplexes are distinguished based on their distinct melting temperatures. Normal placental DNA was used as negative control, and DNA from cell lines with known *N-ras* mutations served as positive control.

2.5. Statistical analysis

Correlation between *B-raf* mutation rate and tumour type was determined by two-tailed Fisher test (STATSOFT 6.0, Tulsa OK, USA). Differences were considered significant at a P value less than 0.05.

3. Results

3.1. Clinico-pathological findings

The clinical features of the patients are summarised in Table 1. The average age of the HTT patients was 44 ± 10 years and the female/male ratio was 4:1 (Table 1). The histopathological features of HTT are presented in Fig. 1.

3.2. B-raf mutations

B-raf exons 11 and 15 were PCR-amplified and subjected to direct sequencing from b oth genomic DNA and total RNA from all of the samples listed in Table 1. Three thyroid cell lines, BHT101 and FB1 (carrying a heterozygous V599E mutation) and 8505C (carrying a homo/hemizygous mutation at the same codon), were used as positive controls. No *B-raf* mutations were detected in HTT, follicular adenoma or nodular goitre samples (Table 1). A heterozygous V599E mutation at both the RNA and DNA level was found in 4 of the 10 PTC samples ($P < 0.003$). P represents comparison of PTC *versus* other tumors. Microscopically, the 4 positive PTC were classic examples of the neoplasm. The sequence chromatograms of one representative negative HTT sample and of one *B-raf*-positive cell line are shown in Fig. 2.

3.3. RET/PTC rearrangements

All of the samples were analysed by RT-PCR for the presence of *RET/PTC1* and *RET/PTC3* rearrangements. The assay was performed with primers designed to flank

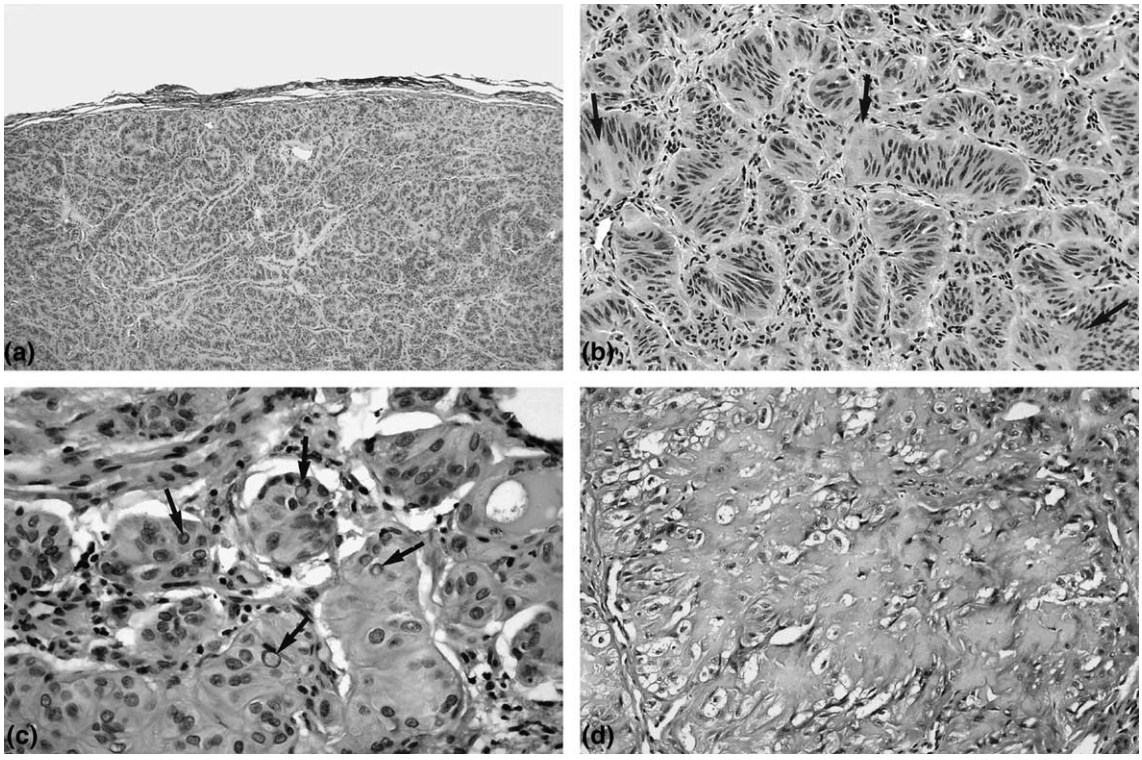

Fig. 1. Histopathology of hyalinizing trabecular tumour of the thyroid. (a) Tumour with trabecular pattern (haematoxylin and eosin (H&E) × 40). (b) Several foci of intra-trabecular hyalinization are visible (arrows) (H&E × 200). (c) Acidophilic material (hyalinization) has replaced most of the cells (right lower). Some nuclei exhibit cytoplasmic inclusions (nuclear "holes") (arrows) (H&E × 400). (d) The trabecular–alveolar pattern is obliterated by abundant acidophilic substance (hyalinization) (H&E × 250).

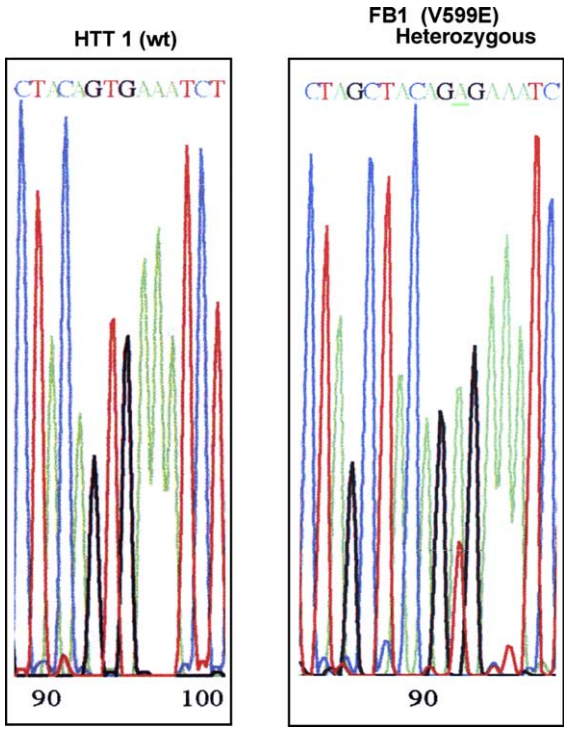

Fig. 2. *B-raf* mutation detection. Sequence chromatograms of exon 15 from one representative HTT patient showing wild-type (wt) *B-raf* sequence and from FB1 cells carrying the 1796 T-A heterozygous transversion (V599E).

the fusion point between *RET* and its partner gene (*H4* or *RFG*) as illustrated in Fig. 3. No *RET*/*PTC* rearrangement was detected in follicular adenomas or nodular goitres. Rearrangements were found in the HTT and PTC series with a prevalence of 13 out of 28 HTT and three out of 10 PTC (Table 1).

3.4. N-ras mutations

Activating point mutations in *RAS* family genes are common in follicular adenomas and carcinomas; they are less common in PTC. Although mutations may be found at each of the three *RAS* genes, *N-ras* mutation at codon 61 is the predominant one [18]. We determined the frequency of *N-ras* 61 mutation. Mutation of *N-ras* were studied at the DNA level. No HTT had *N-ras* mutations, while one of 10 follicular adenomas had a CAA to CGA transition at codon 61 (Table 1).

4. Discussion

In this study, we provide a detailed characterisation of the genetic background of HTT with respect to *RET*/*PTC* rearrangements, and *B-raf* and *N-ras* mutations. Our results demonstrate the absence of *B-raf* and *N-ras* point mutations in 28 cases of HTT. By adding to our series a previously published analysis on a small HTT sample set (five cases), we can conclude that out of 32 HTT samples no sample was *B-raf*- positive. By contrast, 13 of 28 HTT showed *RET*/*PTC* rearrangements. In HTT, we found a higher incidence of the *RET*/*PTC1* variant (10 out of the 13 positives) compared with the *RET*/*PTC3* variant. These results are intriguing be-

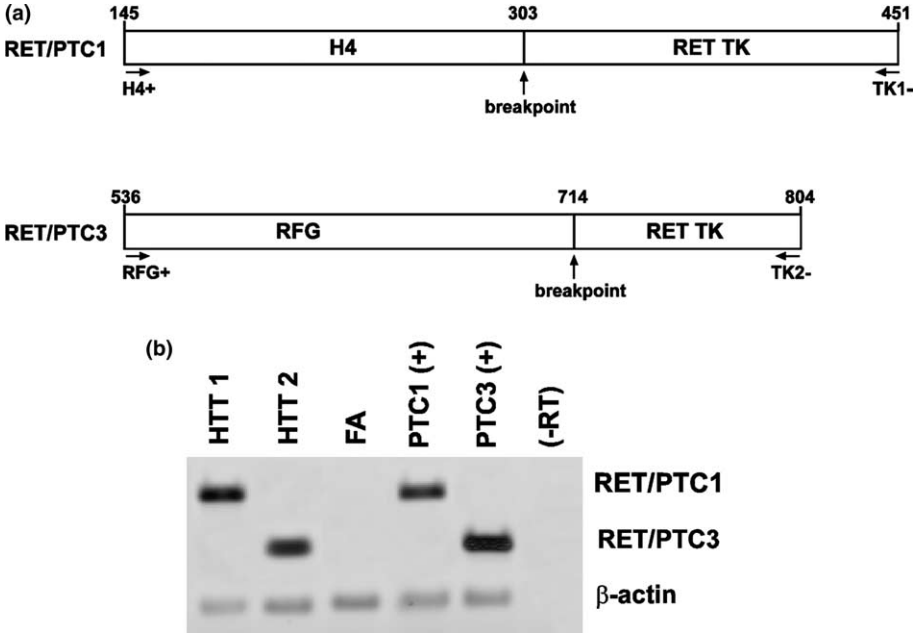

Fig. 3. *RET*/*PTC* rearrangements detection. (a) Schematic graph of H4-RET (*RET*/*PTC1*) and RFG-RET (*RET*/*PTC3*) fusions; breakpoints, oligonucleotides and nucleotide positions are indicated. (b) RT-PCR analysis of *RET*/*PTC1* and *RET*/*PTC3* expression in representative patients and positive control tumours. (−RT): sample HTT1 amplified without previous reverse transcription.

cause of the association of *RET/PTC3* with aggressive solid-follicular [24] and tall-cell [25] PTC variants.

Although HTT shares with PTC a high prevalence of *RET/PTC* rearrangements, it did not show the point mutations in *B-raf* that were frequently present in PTC (nor the *N-ras* mutations that may also be found in PTC). Other functional differences between the two proteins have emerged. *B-raf* mutations have been found in anaplastic thyroid carcinomas [26], while *RET/PTC* rearrangements were absent in these highly malignant tumours [27]. To reconcile these findings, we hypothesise that the biological outcome of the activation of a particular signalling protein (*B-raf*, for example) is different when the protein is activated by a point mutation (as occurs in PTC) versus a natural upstream regulator (*RET/PTC*, in the case of PTC and also HTT). In particular, the extent and kinetics of activation and parallel stimulation of other pathways might be very different in the two conditions, thereby determining the final biological response. In the case of HTT, the identification of the oncogene(s) activated in *RET/PTC*-negative cases, as well as of the biochemical pathway(s) in which they function, will be of great help in discriminating between the different possibilities.

Conflict of interest statement

None declared.

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