High Growth Rate of Benign Thyroid Nodules Bearing *RET/PTC* Rearrangements

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Context: Benign thyroid nodules display a broad range of behaviors from a stationary size to a progressive growth. The *RET/PTC* oncogene has been documented in a fraction of benign thyroid nodules, besides papillary thyroid carcinomas, and it might therefore influence their growth.

Objective: The aim of the present work was to evaluate whether *RET/PTC* in benign thyroid nodules associates with a different nodular growth rate.

Study Design: In this prospective multicentric study, 125 subjects with benign nodules were included. *RET* rearrangements were analyzed in cytology samples; clinical and ultrasonographic nodule characteristics were assessed at the start and at the end of the study.

Results: *RET/PTC* was present in 19 nodules. The difference between the mean baseline nodular volume of the *RET/PTC*− and *RET/PTC*+ nodules was not significant. After 36 months of follow-up, the *RET/PTC*+ group (n = 16) reached a volume higher than the *RET/PTC*− group (n = 90) (5.04 ± 2.67 *vs.* 3.04 ± 2.26 ml; $P = 0.0028$). We calculated the monthly change of nodule volumes as a percentage of baseline. After a mean follow-up of 36.6 months, the monthly volume increase of nodules bearing a *RET* rearrangement was 4.3-fold that of nodules with wild-type *RET* (1.83 ± 1.2 *vs.* 0.43 ± 1.0% of baseline volume; $P < 0.0001$).

Conclusions: Benign thyroid nodules bearing *RET* rearrangements grow more rapidly than those with wild-type *RET*. Searching for *RET* rearrangements in benign thyroid nodules might be useful to the clinician in choosing the more appropriate and timely therapeutic option. **(*J Clin Endocrinol Metab* 96: E916–E919, 2011)**

Nontoxic solitary and multinodular goiter is a common disease characterized by the presence of one or more nodules with a normal thyroid function. The use of fine-needle aspiration cytology (FNAC) and the application of ultrasonography resulted in an earlier and more accurate diagnosis of thyroid nodules. The management of non-complicated benign nodules includes follow-up without treatment or medical treatment aimed at suppressing TSH secretion. In routine clinical practice, benign nodules showing high growth rate are usually treated surgically; thus, it would help to be able to detect benign nodules with a higher growth rate potential to direct patients to a timely appropriate treatment. Although the natural history of nodular goiter is that of a gradually increasing size, it is difficult to predict how fast a nodule will grow in a given patient because no specific growth parameters exist, and

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Abbreviations: FNAC, Fine-needle aspiration cytology; PTC, papillary thyroid carcinoma.

the optimum management remains controversial (1). Identifying genetic alterations predictive of higher growth rate for benign nodules could lead to a more appropriate management of the disease.

Rearrangements of the *RET* protooncogene generate chimerical cytoplasmic kinases (RET/PTC) that stimulate thyroid cell proliferation and the development of papillary thyroid carcinomas (PTC) (2, 3).

The *RET/PTC* oncogene has also been documented in benign thyroid nodules (4–6). It is possible that when expressed, this oncogene promotes thyroid cell growth and the enlargement of benign nodules.

In this study, we have performed genetic and ultrasound evaluation of patients to find out whether the expression of *RET/PTC* is associated with faster growing benign thyroid nodules.

Patients and Methods

Patients and study design

This is a multicentric prospective study involving 125 consecutive outpatients with nodular goiter. Patients entered the study, following approval from the institutional review boards, after giving written consent. Entry criteria included: thyroid nodules with clear measurable diameters and greatest diameter equal to or greater then 10 mm; ultrasonographic characteristics of a solid nodule; cytology consistent with a Thy 2 colloid nodule according to cytological classification from the British Thyroid Association; and normal free T_4, free T_3, and TSH levels. When more nodules were present (50.4% of the patients), only the largest one was taken into consideration for the study. Exclusion criteria were: TSH suppressive therapy, possible presence of autonomous areas within the thyroid as indicated by either TSH levels below the lower normal limit or hyperfunctioning areas at scintiscan, after menopausal hormone replacement therapy.

Ultrasonography

For each patient, thyroid ultrasonography was performed at the same center: at Presidio Ospedaliero "Umberto I" (Turin, Italy) using a 7.5- to 10-MHz linear transducer (Esaote, Genoa, Italy) or at University Federico II (Naples, Italy) using a General Electric RF 3600 instrument with a 7.5-MHz linear transducer (GE Medical Systems, Torino, Italy). In both centers, the variation coefficient among examiners, estimated by performing repeated measurements on the same subject, was about 8%. The nodule volume was calculated according to the formula of the ellipsoid model: volume (ml) = width × length × thickness × $\pi/6$.

Fine-needle aspiration

The aspirated material was used for cytological examination; the remaining portion was dispersed into TRI-reagent buffer (Sigma, St. Louis, MO) and stored at −20 C until RNA extraction. Three independent pathologists blindly reviewed cytology findings. Samples were classified according to cytological classification from the British Thyroid Association and the UK Royal College of Physicians (7).

Detection of *RET/PTC* rearrangement

Total RNA was extracted using TRI Reagent, resuspended in 10 μl diethyl pyrocarbonate-treated water, and reverse-transcribed with SuperScript III (Invitrogen, Milan, Italy) in a 20-μl reaction volume with random primers. RNA integrity and the efficiency of the reverse-transcribed reaction were confirmed by RT-PCR for thyroglobulin mRNA. *RET/PTC-1* and *RET/PTC-3* were detected by Southern blot on RT-PCR products as described, using appropriate primers and oligoprobes specific for the *TK* domain, *H4*, or *ELE1* (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org) (8). The PCR products of four samples positive for *RET/PTC* were sequenced to demonstrate that the PCR amplified a chimerical cDNA containing *H4* or *ELE1* and the exon 12 of *RET*.

Statistical analysis

Comparisons between quantitative variables of the *RET/PTC*-positive and -negative groups were based on Wilcoxon two-sample test. Comparisons between basal and final nodule volumes were based on Wilcoxon signed-rank test. Analysis for multiple comparisons was conducted using Bonferonni's adjustment with correction for correlation between observations. Four pairwise comparisons were made, and significance for adjusted differences was set at $P \leq 0.0125$.

Results

A total of 125 nodules with Thy 2 FNAC entered the study. *RET* rearrangement was demonstrated in 19 nodules, three *RET/PTC-1*, and 16 *RET/PTC-3*. Clinical and laboratory characteristics of the patients are reported in Supplemental Table 2. Ultrasonographic measurements of nodule volumes were performed every 6 months or yearly, for a follow-up of at least 36 months. Three patients in the *RET/PTC*+ group ended the study earlier (after 7, 9, and 11 months) because surgery was needed for a rapid, progressive enlargement of the nodule. For all three cases histopathology was compatible with a benign hyperplastic nodule. Of the *RET/PTC*− group, three patients dropped out the study before 36 months, and 13 were followed up for more that 36 months. At the end of the study, the mean follow-up was 30.9 ± 10.9 months for *RET/PTC*+ and 36.9 ± 3.5 months for *RET/PTC*− nodules.

Nodule volume variations at 36-month follow-up

Ninety *RET/PTC*− and 16 *RET/PTC*+ nodules were followed for 36 months (Table 1 and Fig. 1, A and B). Mean baseline nodular volumes of the *RET/PTC*− and *RET/PTC*+ nodules were comparable (2.62 ± 1.65 and 3.04 ± 2.26 ml), as were patients' ages at FNAC (52.9 ± 6.8 and 53.7 ± 11.2 yr) and female/male ratio (73/17 and 12/4, respectively). There were no other significant differences between the two groups.

After 36 months of follow-up, the *RET/PTC*+ group reached a higher nodular volume (5.04 ± 2.67 *vs.* 3.18 ±

TABLE 1. Nodule volume variations after 36 months of follow-up

	RET/PTC−	*RET/PTC+*	*P*
n	90	16	
Baseline volume			
Mean	2.62	3.04	0.7307
Median	2.21	2.37	
SD	1.65	2.26	
Final volume			
Mean	3.18	5.04	0.0028
Median	2.83	4.37	
SD	2.11	2.67	
Baseline *vs.* final volume			
P	0.0011	<0.0001	

2.11 ml; $P = 0.0028$). Nodule volume increase of the RET/PTC+ group was significant ($P < 0.0001$), as was that of the *RET/PTC−* group ($P = 0.0011$). To compare nodules with a very different baseline volume that ranged from 1.30 to 10.29 ml, the volume change over a 36-month follow-up was calculated for each nodule as a percentage baseline variation (Fig. 1C). The volume increase of the *RET/PTC+* nodules (n = 16) was 3.6-fold that of the *RET/PTC−*.

Monthly nodule volume variations

The exclusion of more rapidly growing nodules that underwent surgery could bias the analysis, reducing the differences between the two *RET/PTC*-negative and -positive groups. To include in the measurements all 125 nodules regardless of follow-up duration (ranging from 7–48 months) and size (ranging from 1.3–10.3 ml), and assuming that the size increase over time was constant, we calculated the monthly percentage baseline change of the nodule volumes according to the formula: $[(\text{final volume}/\text{baseline volume})^{(1/\text{months of follow-up})} - 1] \times 100$. Through this analysis, the higher growth rate of the nodules bearing *RET/PTC* became even more evident (Fig. 1D). The monthly volume increase of nodules bearing a *RET* rearrangement was a 4.3-fold that of nodules with wild-type *RET* (1.83 ± 1.2 *vs.* 0.43 ± 1.0%; $P < 0.0001$).

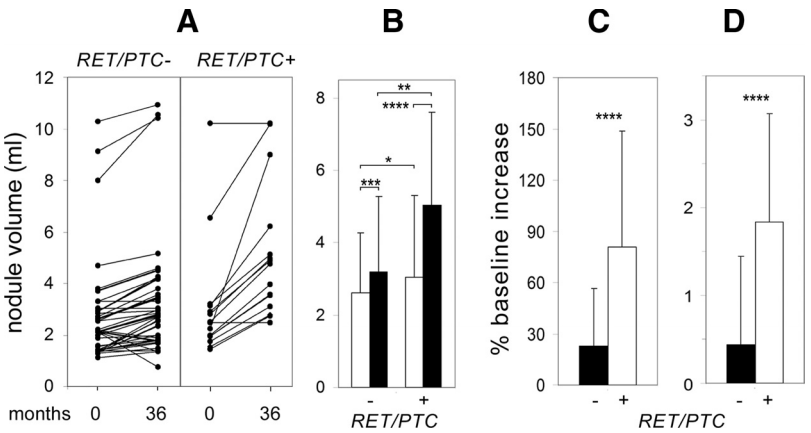

FIG. 1. Changes and relative increase of nodule volume as determined by ultrasonography. A, Volumes at baseline and after 36 months of follow-up. B, Mean and SD of nodule volumes at baseline (*empty bar*) and after 36 months (*full bar*). C, Mean and SD increase of nodule volumes, reported as the percentage baseline increase of individual nodules, over a follow-up of 36 months. D, Mean and SD of percentage monthly baseline increase of nodule volumes, calculated as reported in the text over a follow-up of 7–48 months. Comparisons between quantitative variables of the *RET/PTC*-positive and -negative groups were based on Wilcoxon two-sample test. Comparisons between basal and final nodule volumes were based on Wilcoxon signed-rank test. *, Not significant; **, $P = 0.002$; ***, $P = 0.001$; ****, $P < 0.0001$.

Discussion

The biological and clinical significance of the presence of *RET* rearrangements in benign thyroid lesions is largely unknown. The oncogene RET/PTC in a benign thyroid nodule might be involved in transformation into carcinoma, or might influence the clinical characteristics of the nodule and its growth. RET/PTC positively controls the MAPK cascade, and ultimately cell proliferation (9). Hence, independent from its ability to transform a benign nodule into a carcinoma, it can stimulate its growth.

Our analysis revealed that benign thyroid nodules bearing a *RET* rearrangement grow more rapidly than those with wild-type *RET*. The volume enlargement of the *RET/PTC+* nodules was very variable (monthly increase from 0 to 3.9% over baseline; mean = 1.88%). The large individual variability may reflect the level of expression of the oncogene. Although the Southern blot on RT-PCR is not a quantitative assay, we observed very different intensities of the Southern blot bands among the samples analyzed (data not shown). It is tempting to speculate that different intensities could reflect variable expression of the oncogene in the samples.

Interphase fluorescence *in situ* hybridization analysis of tissues from papillary thyroid tumors demonstrated that *RET/PTC* rearrangements can occur only in a fraction of the cells (10, 11). Similarly, it is also possible that the *RET/PTC+* benign nodules are composed of a mixture of cells, with only a few of them bearing the oncogene. Even if *RET/PTC* is present only in a subset of cells, it can influence the entire nodular mass or a large part of it. Chemokines are small cytokines that, through a seven-domain transmembrane receptor, activate the MAPK pathway and ultimately stimulate cell proliferation (12, 13). Expression of the chemokines CXCL1 and CXCL10 and their corresponding receptors is induced by RET/PTC that thus modulates cell proliferation also by an autocrine/paracrine mechanism

(14, 15). Therefore, a paracrine loop may stimulate proliferation not only of the cells bearing the oncogene but also of neighboring cells, involving a larger part of the nodule.

The variable volume changes observed could also be related to the heterogeneity of the patients included in the study. It is noteworthy that despite this heterogeneity, the differences between the two groups were indeed highly significant. Future analysis should consider separate groups for gender, age, and especially the menopausal status of the patients because slow growth of benign thyroid nodules in postmenopausal women has been reported (1, 16, 17).

In summary, this study demonstrates that the RET/PTC oncogene is a factor associated with a faster volume increase in benign nodules. At the current status of knowledge, because of great individual variability and the involvement of different factors, it is very difficult to predict how fast and to what extent a benign thyroid nodule will grow (17–20). Even an accurate follow-up by ultrasonography can take years to answer this question because of the variability of the measurements and the overall slow changes. The search for *RET* rearrangements in benign nodules may provide a mean of identifying those predisposed to a faster enlargement. This may help the clinician to choose the more appropriate and timely therapeutic option.

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