

Assessment of RET/PTC Oncogene Activation in Thyroid Nodules Utilizing Laser Microdissection Followed by Nested RT-PCR

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Summary

Single palpable nodules of the thyroid gland are common in clinical practice; the majority of such lesions are benign. However, noninvasive thyroid nodules that exhibit borderline morphological signs of papillary cancer represent a diagnostic challenge. Rearrangements of the RET oncogene have been proposed as a marker for papillary thyroid cancer. In this chapter, methods for the analysis of the RET oncogene in laser microdissected papillary thyroid cancer tissue are described.

Key Words: Oncogene; RNA extraction; thyroid neoplasm.

1. Introduction

Solitary palpable nodules of the thyroid gland are frequently encountered in clinical practice with an incidence in the United States of 2–4% among adults. Females are more commonly affected than males. The majority of such lesions are benign processes, either follicular adenomas or non-neoplastic conditions such as nodular hyperplasia (colloid nodules), simple cysts, or thyroiditis, including patients with Hashimoto's thyroiditis (1–4). Noninvasive thyroid nodules that exhibit borderline morphological signs of papillary cancer are difficult to diagnose and represent a challenge to the pathologist that will establish the final diagnosis, eventually leading to an accurate medical intervention (5). In these cases, the morphological signs of papillary carcinoma are incomplete, in terms of “quality” when the cytologic alterations are not convincing enough and/or “quantity” when they are not uniformly present throughout the nodule. Regardless, they are superimposed on thyroid nodules that have otherwise benign histologic features and either a well-defined tumor capsule—as in the

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case of follicular adenomas—or a poorly defined or incomplete capsule, as in the case of hyperplastic nodules. Rearranged versions of the RET proto-oncogene called RET/PTC (for papillary thyroid carcinoma) (6) are a marker for papillary thyroid cancer (7). RET/PTC results from the fusion of the RET tyrosine-kinase (TK) domain with the 5' terminal region of heterologous genes, which leads to the formation of RET/PTC chimeric oncogenes. To date, at least 16 such chimeric mRNAs involving 11 different genes have been reported, of which RET/PTC1 (resulting from the fusion of RET with the H4 gene) and RET/PTC3 (resulting from the fusion of RET with the RFG gene) are by far the most common (6,8). Reverse transcriptase-polymerase chain reaction (RT-PCR) for RET/PTC1 and RET/PTC3 can be performed successfully on RNA extracted after laser capture microdissection (LCM) of thyroid tissue. Evaluation of thyroid nodules exhibiting borderline histological morphology for papillary thyroid carcinoma and analysis of RNA obtained after LCM demonstrates that these lesions often harbor rearranged RET alleles and that RET/PTC activation is restricted to those areas where the classical cytological papillary carcinoma features (nuclear clearing, overlapping, and contour irregularities in the form of indentations, grooves, and pseudo-inclusions) are better developed (9). It may be clinically useful to perform LCM for RET/PTC transcript analysis in thyroid nodules with incomplete papillary thyroid carcinoma cytologic alterations. Cases with detectable RET/PTC oncogene activation should be considered as papillary carcinoma when the aberrant transcripts are detected after LCM of areas with *well-developed* cytologic changes, no matter how focal these changes may be within the thyroid nodule. In fact, these cases fulfill both morphologic (presence of the diagnostic alterations) and molecular (aberrant RET/PTC transcripts) criteria for such a diagnosis. The diagnosis of cancer, however, should be limited to these areas and it is not correct to consider the entire nodule as papillary cancer, an observation that is clinically relevant since tumor size influences thyroid carcinoma staging (10). Cases in which the PTC cytologic alterations are only *poorly developed* and focal are more difficult to understand. One may argue that if the molecular markers of papillary carcinoma are there, these lesions should be considered malignant as well. Since cells with RET rearrangement represent a clone (or a subclone) within the nodule (9), its “size” (i.e., the relative proportion of the nodule with the aberrant RET/PTC transcripts) should probably also be taken into account. On the other hand, the finding of tumor-specific molecular alterations *per se*, without full support of clinicopathologic data, does not necessarily imply malignancy, and the term “well differentiated tumor of uncertain malignant potential” appears justified for these lesions, also considering that we know little about their clinical behavior (11). Limited follow-up information indicates that an extremely good prognosis has to be expected, but it is possible that these

noninvasive thyroid nodules with focal RET/PTC transcriptional activation and poorly developed cytologic changes represent a new class of papillary carcinoma precursor.

2. Materials

2.1. LCM and RNA Extraction

1. Serial 5- μ m sections of paraffin-embedded tissue.
2. Plain, nonadhesive glass slides.
3. Methyl green.
4. PixCell I system (Arcturus Engineering, Mountain View, CA).
5. Thermoplastic film-coated cap.
6. Eppendorf tube.
7. Proteinase K (Sigma Chemical, St. Louis, MO).
8. Guanidinium thiocyanate.
9. β -Mercaptoethanol.
10. 0.5% Sarkosyl.
11. Tris-HCl, pH 7.5.
12. 2 M Sodium acetate, pH 4.0.
13. Water-saturated phenol.
14. Chloroform-isoamyl alcohol.
15. Glycogen solution (10 μ g/ μ L).
16. Isopropanol.
17. Ethanol.
18. DNase.
19. Random hexamers.
20. Murine leukemia virus (MuLV) RT.
21. MgCl₂.
22. dNTP.
23. RNase inhibitor.
24. PCR buffer II (Perkin-Elmer, Foster City, CA).
25. Primers specific for the human aldolase gene (for mRNA control).
26. AmpliTaq DNA polymerase.
27. Perkin-Elmer 9700 thermal cycler.
28. 3% Agarose gel.
29. Probe covering the tyrosine-kinase domain of RET.
30. Previously characterized papillary carcinoma samples (for positive control).
31. Undifferentiated thyroid carcinoma cell line ARO that lacks RET/PTC rearrangement.

3. Methods

3.1. LCM and RNA Extraction

Cases with discrete foci of PTC-type cytologic alterations can be processed for RNA extraction and nested RT-PCR after LCM (**Fig. 1**) following the gen-

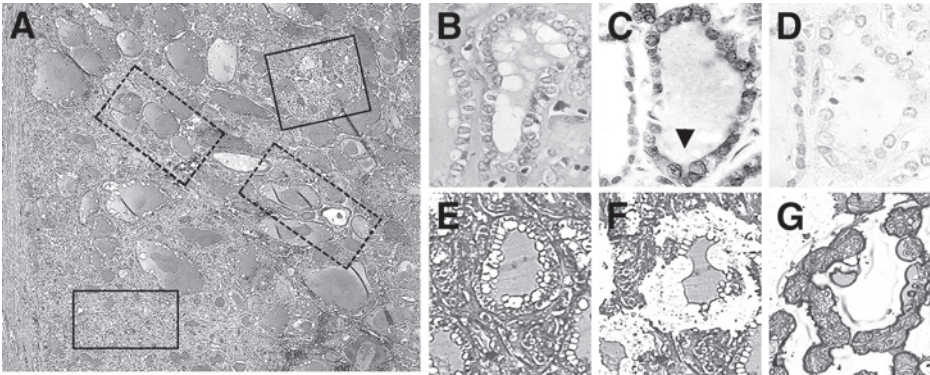


Fig. 1. Histologic appearance, immunohistochemical staining with RET(TK) antibodies, and laser capture microdissection of tissue from one noninvasive thyroid nodule with poorly developed papillary thyroid carcinoma-type nuclear changes (9). In the low-magnification image (A) the rectangles with solid lines indicate the areas of the tumor with papillary carcinoma features while the rectangles with dotted lines indicate the areas lacking them. The areas with and those without papillary carcinoma features were targeted for laser capture microdissection and separately processed for RNA extraction. A higher magnification of the foci with papillary carcinoma features is shown in (B). Cells with cytologic alterations of papillary carcinoma were immunohistochemically positive with RET(TK) antibodies (C); the corresponding negative control is shown in (D). The nuclear features of papillary carcinoma are poorly developed with minor degrees of nuclear clearing as seen in B, C, D, and occasional grooves (C) (arrowhead). The cells with nuclear alterations were positioned for laser capture microdissection (E), selectively captured (F), and visualized on the thermoplastic coated caps (G) before being processed for RNA extraction. Laser capture microdissection was similarly performed in areas of the nodule lacking the cytologic alterations of papillary carcinoma. RET/PTC3 transcripts were detected after nested RT-PCR of RNA extracted from areas with cytologic alterations but not after nested RT-PCR of RNA extracted from areas lacking cytologic changes. (Reproduced from ref. 9 with permission.)

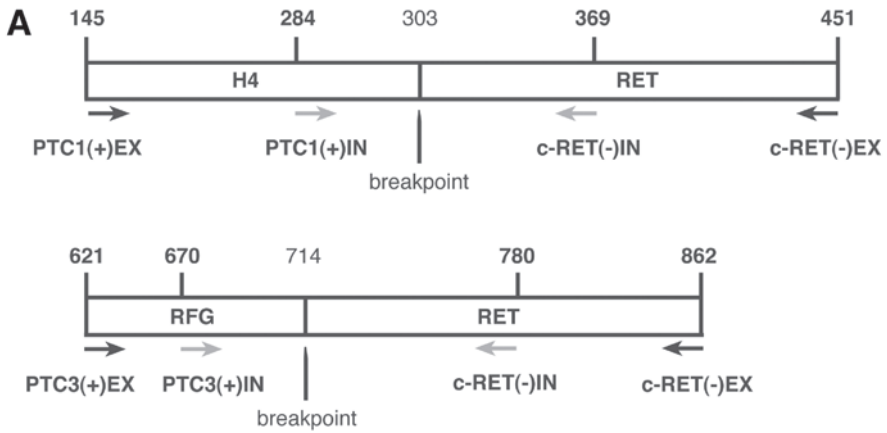
eral procedures outlined at the National Institute of Health LCM web site (<http://dir.hichd.nih.gov/lcm/lcm.htm>).

1. Serial 5 μ m sections are mounted on plain nonadhesive glass slides, dewaxed, and stained with methyl green.
2. The microtome and the water bath should be decontaminated before cutting in each case.
3. The number of serial sections will depend on the amount of material available (usually four to eight sections will suffice).
4. Areas from the same thyroid nodule lacking papillary carcinoma-type nuclear changes can also be targeted for LCM and used as negative controls.

5. Approximately 1000 30- μm shots are used to transfer on the thermoplastic film-coated cap cells obtained from each thyroid lesion targeted for microdissection.
6. RNA is extracted according to established protocols (*see* **Notes 1** and **2**).
7. Briefly, each cap is placed in an Eppendorf tube containing 200 μL of 6 mg/mL proteinase K, 1 M guanidinium thiocyanate, 25 mM β -mercaptoethanol, 0.5% Sarkosyl, and 20 mM Tris-HCl, pH 7.5.
8. The Eppendorf tube should be inverted multiple times to fully digest the tissue off the cap.
9. Twenty μL ($0.1 \times \text{vol}$) of 2 M sodium acetate, pH 4.0, and 220 μL ($1 \times \text{vol}$) of water-saturated phenol are added to the RNA extraction solution followed by chloroform-isoamyl alcohol ($0.3 \times \text{vol}$).
10. After vigorous vortexing and cooling on wet ice, the samples are centrifuged to separate the aqueous and organic phases.
11. The aqueous phase is transferred to a new tube containing 1 μL of glycogen solution (10 $\mu\text{g}/\mu\text{L}$) used as a carrier and to facilitate pellet visualization.
12. After adding an equal volume of cold isopropanol the RNA is precipitated at -20°C overnight, centrifuged, washed with ethanol, treated with DNase, and reextracted.
13. The pellets should be stored at -80°C .

3.2. Nested RT-PCR for RET/PTC1 and RET/PTC3 Rearrangements

1. Three μM of resuspended RNA from each case to be analyzed should be reverse-transcribed with 2.5 μM of random hexamers in a 20- μL reaction mix containing 2.5 U/ μL murine leukemia virus (MuLV) RT, 5 mM MgCl_2 , 1 mM each dNTP, and 1 U/ μL RNase inhibitor in 1X PCR buffer II.
2. The thermoprofile for cDNA generation used is 25°C for 10 min, 42°C for 60 min, 99°C for 5 min, and 5°C for 5 min.
3. RT-PCR with primers specific for the human aldolase gene should be used for mRNA control.
4. The aldolase + primer is 5'-CGC AGA AGG GGT CCT GGT GA-3' (nucleotides 18 to 37 of exon 1), the aldolase – primer is 5'- CAG CTC CTT CTT CTG CTC CG-3' (nucleotides 175 to 194 of exon 2) (**I2**).
5. The expected 176-bp product for aldolase should be obtained to verify RNA integrity.
6. RET/PTC1 and RET/PTC3 transcripts are investigated using nested RT-PCR. The primer sequence and location are shown in **Fig. 2**.
7. For PCR, 3 μL of the cDNA template are used for the first round of amplification with the external primer sets (**Fig. 2**) in a 30- μL reaction volume with 0.1 μM for each primer, 200 μM each dNTP, and 0.8 U AmpliTag DNA polymerase in Buffer II containing 2.0 mM MgCl_2 .
8. After a 12-min hot start at 94°C , nine cycles of touchdown amplification are performed (progressively lowering the annealing temperature from 61°C to 55°C), followed by 40 cycles of amplification (94°C for 30 s, 55°C for 45 s, and 72°C for 45 s) with a Perkin-Elmer 9700 thermal cycler.



B

PRIMERS NAME	SEQUENCE	PRIMER SIZE	EXPECTED PRODUCT SIZE
PTC1(+)-EX	5'-ATT GTC ATC TCG CCG TTC-3'	18 mer	307 bp
PTC3(+)-EX	5'-AAG CAA ACC TGC CAG TGG-3'	18 mer	242 bp
c-Ret(-)-EX	5'-CTT TCA GCA TCT TCA CGG-3'	18 mer	downstream primer
PTC1(+)-IN	5'-GCA AAG CCA GCG TTA CC 3'	17 mer	86 bp
PTC3(+)-IN	5'-CCC CAG GAC TGG CTT ATC 3'	18 mer	111 bp
c-Ret(-)-IN	5'-TTC GCC TTC TCC TAG AGT 3'	18 mer	downstream primer

Fig. 2. (A) Schematic of the nested primer location relative to RET/PTC1 and RET/PTC3. Primer positions are according to the RET/PTC1 (5, GB no. M31213) and RET/PTC3 (7, GB no. X77548) standard sequences with the numeration beginning from the start codon. (B) Primer sequence and expected size of the amplicons for nested RT-PCR. EX: external primer set. IN, internal primer set. (Reproduced from ref. 9 with permission.)

9. For the second round of amplification, 2 µL of first-round PCR product are used with the internal primer sets (Fig. 2) and the same reaction conditions described for the first amplification round.
10. The nested RT-PCR products for RET/PTC1 and RET/PTC3 are analyzed on a 3% agarose gel and hybridized with a probe covering the tyrosine-kinase domain of RET (13).

11. RNA extracted from previously characterized papillary carcinoma samples can be used as positive controls.
12. Amplification in the absence of RT, or in the presence of RNA extracted from the undifferentiated thyroid carcinoma cell line ARO that lacks RET/PTC rearrangement, can be used as a negative control (**13,14**).

4. Notes

1. When using this technique, especially in wax-embedded 10% neutral-buffered formalin-fixed archive material, the integrity of the mRNA can be compromised to a point that no suitable RNA will be available for the assay. In our experience, amplification of a housekeeping gene (aldolase) is successful in approximately two thirds of LCM microdissected tissue from formalin-fixed, paraffin-embedded routinely processed samples of thyroid tissue. Because RNA from such specimens is significantly degraded compared with snap-frozen material, target amplicons involving small stretches of RNA sequence are more successful than larger ones (**15,16**).
2. Although a number of methods for extracting RNA are available, proteinase K digestion of microdissected tissue is an essential step (**16**). Histological dyes bind to cellular components such as DNA and RNA, and can also interfere with nucleic acid retrieval. Cellular RNA appears to be particularly more sensitive and dependent on the histological stain employed (**15**). Methyl green does not bind to RNA (**17**) and is a valid alternative to hematoxylin or toluidine blue for RNA expression analysis of thyroid tissue.
3. Precipitating fixatives such as ethanol improve preservation of nucleic acids (**16**) and thyroid samples are no exception to this general rule; however, the overwhelming majority of archival material in surgical pathology practice is formalin-fixed. Published protocols are available to optimize nucleic acid recovery from LCM microdissected tissue (**15**).

It is important to keep in mind that although fixed and frozen specimens render different amount and quality of nucleic acid, pre-LCM tissue preparation and microdissection itself are influenced by the type of starting material (**16**). A recent work designed to detect RT-PCR products from paraffin-embedded tissues, using different fixatives, points out that the method employed to detect the RT-PCR product obtained from wax-embedded tissues may also influence the result. In fact, real-time RT-PCR analysis shows a higher success rate in RNA recovery compared with ethidium bromide stain of conventional agarose gels, although the size of the amplicon may also be a factor (**18**). In summary, as synthesized by Goldsworthy et al., “shortening the duration of fixation, increasing the size sample, amplifying products less than 100 bp, using a nested primer method, and increasing the number of amplification cycles all can be used to achieve positive results” (**18**) and these general concepts fully apply to the recovery of RNA from thyroid tissue samples.

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