CLINICAL STUDY

Medullary thyroid carcinoma: an accurate pre-operative diagnosis by reverse transcription-PCR

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Abstract

Objective: To study the expression of calcitonin (CT) and thyroglobulin mRNA in samples of leftover cells in needles used for fine-needle aspiration biopsy either from thyroid tumours or cervical lymph nodes.

Patients and methods: Specimens were analysed using reverse transcription-polymerase chain reaction; 12 samples from 11 patients were included and molecular diagnosis was compared with cytological or histological diagnosis and serum CT measurements.

Results: Transcripts of the CT gene were detected in all six patients with medullary thyroid carcinoma (MTC) but in none of the other patients.

Conclusions: Present data reinforce this technique as a reliable and alternative tool to establish the pre-operative diagnosis of MTC, especially when cytological examination is not conclusive or when cytological information is not in agreement with clinical data. Furthermore, it may be clinically useful to identify those conditions in which increased serum CT in the presence of a thyroid nodule is not due to MTC.

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Introduction

The diagnosis of medullary thyroid carcinoma (MTC) or of any other type of thyroid tumour depends mainly on the results of cytological and histological examinations. Calcitonin (CT) and carcinoembryonic antigen (CEA), secreted by MTC cells, may be useful biochemical markers for diagnosis. However, they are not routinely assessed during the evaluation of thyroid cold nodules.

In some situations, cytological examination may not provide a conclusive diagnosis. In such cases a molecular-based approach may be used, provided there are messenger RNAs (mRNAs) that are cancer-specific.

Thyroid cancers overexpress a number of genes, such as those for epidermal growth factor receptor (1), c-erbB (2), insulin-like growth factor-I (3), p53 (4), dipeptidyl aminopeptidase IV (5), c-Met (6), and CD44E (7). However, as the expression of these genes is not restricted to cancer cells they have little diagnostic potential.

Expression of RET proto-oncogene, CT and CEA are restricted to MTCs (8–10). A new technique of aspiration biopsy-reverse transcription (RT)-PCR aimed at specific mRNAs was established for the pre-operative diagnosis of thyroid carcinomas (11, 12).

Moreover, Takano et al. (13) have demonstrated that MTCs may be diagnosed by RT-PCR by mainly using aspirates from surgically dissected thyroid tissues.

Herein, we performed fine-needle aspiration biopsy (FNAB) in 11 patients with thyroid nodules or cervical lymph nodes and performed RT-PCR to amplify CT and thyroglobulin (Tg) cDNA. The results were compared with cytological and histological information.

Patients and methods

Nine thyroid tissue samples and three cervical lymph nodes samples (Table 1) were obtained by FNAB, using a syringe with a 22 gauge needle. After preparation of a slide for cytological examination, leftover cells were washed out of the needle with a solution containing 400 μl of the lysis buffer used for RNA isolation + 4μl β-mercaptoethanol and kept on ice. Immediately after, RNA isolation was carried out with the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s recommendations.

The whole RNA extracted by FNAB was reverse transcribed with SuperScript (Life Technologies, Inc.) in a 20μl reaction volume with random primers. The
integrity of the RNA and efficiency of the RT reaction in each sample was tested by PCR for the housekeeping gene GAPDH as previously described (14, 15). Thereafter, 1 µl first-strand cDNA was used as template for the PCR reaction with specific primers and under conditions previously described (13) for both CT and Tg. Reaction products were separated electrophoretically on 10% polyacrylamide gels and detected by ultraviolet visualization after ethidium bromide staining.

CT was assayed by immunoradiometric assay using a commercial kit (ELISA-hCT, CIS Biointernational, Gif-sur-Yvette, France). Most normal individuals have values below 10 ng/l; the pentagastrin stimulation test is considered positive when the difference between maximum peak and basal level ratio (Δ) is > 30 ng/l (16).

We looked for germline mutations in the RET proto-oncogene in all MTC patients and in one patient presenting with a follicular tumour and a positive pentagastrin test. High molecular weight DNA from white blood cells was prepared by a manual method adapted from Bowtell (17). RET exons 10, 11, 13, 14, 15 and 16 were amplified using primers previously described (18, 19). Mutational analysis was performed by direct sequencing of purified PCR products, using the Sequenase Version 2.0 kit (USB, Cleveland, OH, USA).

Immunostaining (streptavidin–biotin peroxidase) using anti-calcitonin antibody (DAKO A576) was performed on formalin-fixed tumour tissue in all cases of medullary carcinoma and on thyroid sections corresponding to case 2.

### Results

RNA extraction and reverse transcription were successfully achieved in all samples. Tg mRNA was detected in all adenomatous goiters, follicular adenomas/carcinomas and papillary carcinomas (Fig. 1; lower panel, lanes 1–6). It was also weakly detected in three of six MTC samples corresponding to aspirates from the primitive tumours (Fig. 1; lower panel, lanes 10–12). Expression of CT was detected in all the samples from MTC patients (Fig. 1; upper panel, lanes 7–12) and in none of the samples from non-MTC patients (Fig. 1; upper panel, lanes 1–6).

All MTC patients presented with high levels of serum CT, and immunostaining for CT was positive in tumour sections of all cases. Screening for germline mutations in RET led to the identification of a C634R mutation in one patient (corresponding to samples 9 and 10) with MEN 2A phenotype.

<table>
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<tr>
<th>Samples</th>
<th>Sex</th>
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</table>

* Samples obtained from the same patient.
On the other hand, all of the non-MTC patients but one (corresponding to sample 2) had serum CT values below 10 ng/l. In this particular case, a basal value of serum CT of 10.5 ng/l and a peak value, after pentagastrin stimulation, of 52.6 ng/l (Δ = 42.1 ng/l) were observed. To further understand these findings, we looked for germline mutations in RET and performed immunostaining for CT. No germline mutations in RET were found; however, 'C'-cell hyperplasia was documented in thyroid tissue adjacent to the follicular adenoma.

**Discussion**

Expression of CT and Tg genes as analysed by RT-PCR using RNA obtained by FNAB appears to be a complementary approach to the diagnosis of MTC by maximizing the information that can be obtained from the technique. In the present study, all the samples from MTC patients expressed CT. Moreover, none of the samples from patients harbouring either benign or malignant lesions of follicular origin expressed CT. Both observations provide evidence for the specificity of this molecular marker. Expression of Tg was observed, as expected, in follicular lesions; nevertheless it does not contribute to a differential diagnosis between benign and malignant lesions. The slight expression of Tg in three cases of MTC was interpreted as a result of contamination, an interpretation strengthened by its occurrence in only thyroid samples and not in lymph node samples.

A positive pentagastrin test, as observed in case 2, does not necessarily mean neoplastic 'C'-cell proliferation. This patient had no family history of thyroid tumours and screening for germline mutations in RET proto-oncogene was, likewise, negative. RT-PCR clearly showed expression of mRNA for Tg and absence of CT expression. Histological analysis defined the tumour as a follicular adenoma, validating the RT-PCR results. Immunostaining results not only explained the CT rise after pentagastrin stimulation but also confirmed that 'C'-cell hyperplasia may be present in individuals who are RET mutation negative.

Whether or not all patients with a thyroid nodule should be routinely screened for serum CT is debatable (20, 21). Nevertheless, caution must be taken when interpreting results as reactive 'C'-cell hyperplasia has been reported in neonates, in the elderly, and in patients with Hashimoto’s thyroiditis and surrounding follicular tumours (22). On the other hand, spuriously elevated serum CT in patients with a thyroid nodule may be secondary to the use of methods unable to distinguish the different molecular weight forms of CT in circulation (23). Furthermore, moderately high levels of serum CT, even in patients with goiter, may have a non-thyroidal origin resulting from a neuroendocrine tumour located elsewhere (24).

Misinterpretation of slightly elevated values of CT may lead to unnecessary procedures. In addition to immunohistochemistry and measurements of serum CT, RT-PCR appears to be a valuable approach to establish the diagnosis of MTC.

**References**


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