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# EARLY DIAGNOSIS BY GENETIC ANALYSIS OF DIFFERENTIATED THYROID CANCER METASTASES IN SMALL LYMPH NODES

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ABSTRACT: We report a PCR-based technique for detecting thyroid cancer metastases in small nodes <1.5 cm diameter by the amplification of thyroid specific transcripts TSH-receptor and thyroglobulin. A 100% correspondence with the histopathological diagnosis was observed in the 41/46 nodes (89%) in which an adequate sample was obtained at fine needle aspiration. The genetic analysis resulted more sensitive and accurate than both the cytological analysis (28% inadequate samples, 17% false negative diagnoses) and the thyroglobulin measurement in the aspirates (39% false negatives). The PCR-based genetic analysis may provide a useful tool for diagnosis and follow-up of thyroid cancer.

A frequent and clinically relevant diagnostic problem in the management of patients with differentiated thyroid cancer (DTC) is the nature of an enlarged neck lymph node. Metastatic lymph nodes of the neck may represent the first clinical sign of differentiated thyroid cancer or, more often, indicate postoperative residual or recurrent disease. Correct assessment of the neoplastic or inflammatory nature of an enlarged neck node is therefore important for management of DTC patients. Early localization and diagnostic assessment of metastatic lymph nodes have been achieved with ultrasound (US) guided biopsy followed by cytological examination (1) and thyroglobulin (Tg) measurement in the aspirates (2). These evaluations, however, may lack sensitivity and specificity in small nodes.

Recently, the use of polymerase chain reaction (PCR) has been suggested to identify circulating malignant cells (3) and to detect micrometastases from colon cancer with oncogene mutation (4,5). Since the neoplastic differentiated thyroid tissue is characterized by the expression of specific genes like the TSH receptor (TSH-R) and Tg (6), the genetic analysis appears suitable for detecting small DTC metastases by the PCR amplification of the TSH-R and Tg transcripts in fine needle aspiration (FNA) specimens.

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## Patients and methods

Forty-six separate nodes from 37 patients presenting with either single or multiple enlarged neck lymph nodes were investigated: 25 patients were in follow-up for DTC (22 papillary, 3 follicular); 12 patients had one single or multiple enlarged nodes of unknown origin. Nodes > 1.5 cm diameter were excluded since the study was aimed to the detection of DTC small metastases. All patients had FNA biopsy under US guidance: an aspirate aliquot was smeared for cytological examination and another was frozen for subsequent PCR analysis. Tg was measured in the needle washout in 22 cases. Histopathological diagnosis with multiple sections of excised lymph nodes was assumed as the correct diagnosis.

Reverse transcriptase reaction and PCR amplifications were performed sequentially with a Perkin Elmer 9600 PCR machine (Perkin Elmer Italy) on mRNA extracted with a mRNA Purification Kit (Pharmacia Biotech, Milan, Italy).

PCR conditions were as follows: cycle 1 at 42°C for 15 min and 95°C for 15 s; 40 cycles at 95°C (1 min), 57°C (1 min) and 72°C (for 30 s the first 15 cycles, 1 min the following 15 and 2 min the last 10 cycles); the last cycle at 72°C for 7 min, (42 total cycles). Aliquots (7.5 µl) of the amplification products were then run on 1.5% Tris-Borate-Etilendiamminetetracetate agarose gel containing ethidium bromide.

TSH-R and Tg thyroid specific genes and also glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a control gene ubiquitously expressed, were analysed. GAPDH presence



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ruled out the possibility of inadequate sampling, a possible inconvenience from very small lymph nodes. Specific mRNAs were detected by using the following 3' and 5' oligonucleotides, according to the published sequences (All from GENOSYS, Cambridge, UK):

TSH-R: 5' primer,
5'-GCTTTTCAGGGACTATGCAATGAA-3'
and 3' primer,
5'-AAGGGCAGTGACACTGGTTTGAGA-3'.
The use of these primers gives a PCR product of 213 base pairs .
Tg: amplification of the Tg gene yielded a 408 base pair DNA product by using: 5' primer,
5'-AGGGAAACGGCCTTTCTGAA-3' and 3' primer,
5'-GTGGAGAAGACGACGATTTC-3'.
GAPDH : amplification of the GAPDH gene yielded a 397 base pair DNA product by using 5' primer,
5'-CACCATCTTCCAGGAGCGAG-3' and 3' primer,
5'-TCACGCCACAGTTTCCCGGA-3'.
The couples of primers encompasse regions of the TSH-R and Tg genes including few introns to exclude the possibility of amplification from genomic DNA.

## Results

At histological examination, the 46 lymph nodes examined resulted in 27 DTC metastases and 19 enlarged lymph nodes either reactive or metastatic from non thyroid tumors. GAPDH amplification was detected in 41/46 samples indicating that even in small nodes an adequate sample can be obtained in 89% of cases. Four out of 5 GAPDH negative specimens were from nodes with a maximum diameter <1 cm. Therefore, inadequate sampling was observed in 4/15 (27%) nodes <1 cm vs. 1/31 (3%) larger nodes (1-1.4 cm).

When an adequate specimen was obtained at FNA (GAPDH+), a 100% ccrrespondence was observed between genetic and histopathological diagnosis. (Fig. 1) Twenty-six DTC metastatic lymph nodes were positive for Tg and TSH-R mRNA expression. Fifteen nodes negative for thyroid tissue (8 inflammatory reactions, 2 Hodgkin's lymphomas, 1 non Hodgkin's lymphoma, 3 laryngeal carcinomas and 1 ovary carcinoma metastasis) , negative for Tg and TSH-R mRNA expression, were also correctly identified by the genetic analysis. Figure 1 shows a series of samples representative of the lymph nodes examined. The accurate diagnosis included the selective identification of metastatic vs. reactive nodes in patients with more than one lymph node available for examination (6 cases).

TSH-R and Tg mRNA analysis gave always identical results, indicating that one single thyroid specific mRNA may be adequate for metastasis detection. (Fig. 1) Our small series, however, does not exclude the possibility that in some (rare) cases malignant dedifferentiation may lead to the missing expression of one or more tissue specific proteins.

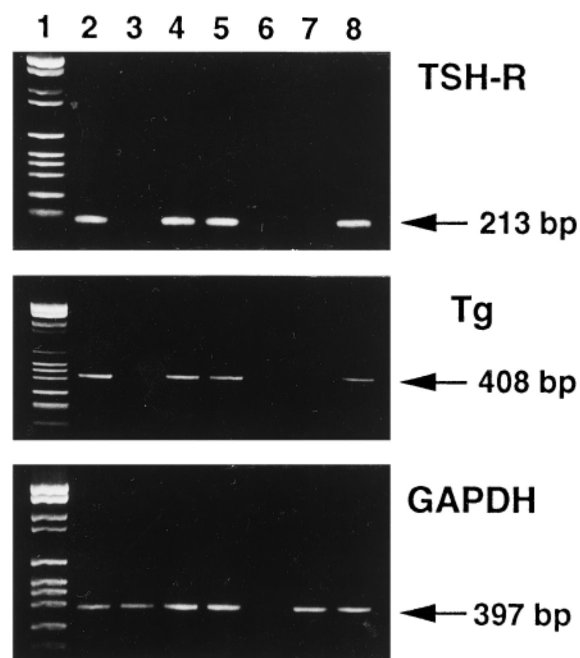


**Figure 1**
Presence of TSH-R, Tg and GAPDH mRNAs Agarose Gel Electrophoresis of PCR amplified DNA in Fine Needle Aspirates from cervical lymph nodes (expected bands for the investigated mRNAs are indicated by arrows).
*Lane 1*: DNA molecular-weight markers. *Lane 2*: Human thyroid cells. Both TSH-R and Tg mRNAs are shown. *Lane 3*: Human lymphocytes. Neither TSH-R or Tg mRNA is present. *Lane 4-5-8*: Lymph node metastases of thyroid carcinomas from 3 different patients. Both TSH-R and Tg mRNAs are detected. *Lane 6*: Inadequate sample from a lymph node. No GAPDH mRNA is detected, indicating that insufficient cellular material was obtained at FNA. *Lane 7*: Lymph node metastasis of a laryngeal cancer. Neither TSH-R or Tg mRNA is present.

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**Table 1** Comparision among genetic analysis (detection of Tg and TSH-R mRNAs), cytology and Tg measurement in FNAB from lymph node metastases < 1.5 cm diameter.

| | Genetic analysis n= 46 % | Cytology n= 46 % | Tg measurement in FNAs n=22 % |
|---|---|---|---|
| Sensitivity | 100 | 83 | 61 |
| Specificity | 100 | 100 | 100 |
| Accuracy | 100 | 85 | 73 |
| Inadequacy | 11 | 28 | = |

The genetic analysis was more sensitive and accurate than the cytological analysis. In the 46 small nodes examined, 13 (28%) inadequate samples were observed at cytology in spite of the fact that the aspirated material was first used for cytological slide smearing and only the remaining aliquot used for the genetic analysis.

The high prevalence of inadequate cytological specimens is a common experience in small lymph node examination and it is confirmed by our experience. Among the 23 adequate samples of histologically proven DTC metastases 4 false negatives (17%) were observed at cytology (Table 1).

The Tg measurement in the FNA needle washout (22 nodes, including 18 DTC metastatic nodes) resulted in 7 (39%) false negatives (Tg value <20 ng/FNA, our cut-off value in histologically proven DTC metastases) indicating that this method may not be sufficiently sensitive for very small metastases. No false positive was observed (Table 1).

## Discussion

The present observations demonstrate the clinical suitability of the molecular analysis for detecting DTC metastases in slightly enlarged neck lymph nodes. This is a further extension of recently reported studies indicating the high sensitivity and accuracy of the PCR amplification technique, for detecting micrometastases in paraffin-embedded specimens, when tissue specific genes or mutations are present (7).

In this regard, DTC appears to be a favorable model for genetic analysis application, in that: a) thyroid tissue is characterized by several tissue specific proteins, such as TSH-R, Tg and thyroid peroxidase; b) total thyroidectomy and $^{131}$I administration eliminate virtually all normal thyroid cells; c) both papillary and follicular DTC histotypes retain many differentiated features of the normal thyroid cell. The present study indicates that in small lymph nodes, PCR-based analysis of thyroid specific mRNAs is a highly sensitive and accurate technique to detect metastases that alternatively can hardly be detected at $^{131}$I scan, US examination, cytology or Tg measurement in the FNA. Lymph node metastases are frequent in DTC and their early detection may affect both the disease progression and the patient overall survival (8). The early diagnosis of residual or recurrent neoplastic disease in the neck lymph nodes by PCR, therefore, may be useful in the clinical management of DTC patients.

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