# Lymph node metastases in papillary thyroid cancer detected by quantitative real-time polymerase chain reaction for thyroglobulin and cytokeratin 19

Krzysztof Kaczka[1], Wojciech Fendler[2], Maciej Borowiec[2], Wojciech Młynarski[2], Agnieszka Celnik[1], Lech Pomorski[1]

[1]Department of General and Oncological Surgery, Medical University of Lodz, Lodz, Poland
 Maria Sklodowska-Curie Memorial Hospital
[2]Department of Pediatrics, Oncology, Hematology and Diabetology, Medical University of Lodz, Lodz, Poland

Papillary thyroid cancer (PTC) metastases in the lymph nodes (LNs) were detected by real-time polymerase chain reaction (PCR) for TG and cytokeratin 19 (CK19), and the obtained results were compared with histopathology. 107 LNs from 34 PTC patients were divided into four blocks by a special cutting device – 2 for histopathology, while the other 2 were tested by quantitative real-time PCR. Metastases were detected in 20 nodes from 10 (29.4%) patients. TG and CK19 expression levels differed vastly between nodes with and without metastatic cells. ddCt of TG in the genetic material extracted from N0 nodes was 9.97 ±4.20, while in nodes with metastases ddCt was 0.91±4.20 ($p < 0.0001$). Cytokeratin 19 showed similar results with expression level (ddCt) in N0 nodes of 10.96 ±2.58 vs. 7.73 ±3.63 in nodes with metastases ($p < 0.0001$). Evaluation of the utility of both parameters showed efficient differentiation of node involvement in the case of TG, with area under the ROC curve (AUC) equal to 0.91 (95% CI: 0.85-0.96). Cytokeratin 19 also allowed for a degree of differentiation but its diagnostic efficacy was lower (AUC 0.76, 95% CI: 0.64-0.88). The combined TG and CK19 quantitative real-time PCR could be used to select a previously missed group of patients with nodal involvement undetectable by standard histopathology.

**Key words:** thyroid cancer, lymph node metastases, quantitative real-time PCR for TG mRNA, quantitative real-time PCR for CK19 mRNA, histopathology.

## Introduction

The impact of neck node metastases on the prognosis of patients with thyroid cancer is uncertain. Although ten-year survival is more than 90%, according to some researchers, it is the worst prognostic factor, which decreases survival rate [1, 2]. Nodal metastases in papillary thyroid cancer (PTC) are frequent (20-50%), and up to 15% of the patients who undergo total thyroidectomy will develop a nodal relapse [3]. Other authors suggest that the presence of metastases in the regional lymph nodes has no clinical value [4-6]. The

extent of lymphadenectomy is also a matter of controversy, especially in cN0 papillary thyroid cancer.

Selective lymphadenectomy or modified, radical lymph node excision could be considered in the case of differentiated thyroid cancer. There have been no randomized trials to date to offer guidance on this issue [7, 8]. Some surgeons suppose that the neck lymph nodes should be evaluated preoperatively and during surgery by physical and ultrasound examination. Only suspected lymph nodes have to be excised [7-9]. However, the 2006 guidelines of the American Thyroid Association recommended consideration of prophylactic bilateral cen-

tral lymph node dissection for all thyroid cancer patients [10]. Total thyroidectomy with central lymphadenectomy does not increase the incidence of main complications: hypocalcemia and vocal cord paresis [11]. Sentinel lymph node biopsy has become more and more common in thyroid surgery but it is not a standard procedure like in breast cancer or melanoma [12-14].

It is necessary to perform many clinical studies to evaluate its prognostic value. We believe that to solve all these controversies there is a need to look for diagnostic methods other than histopathology. It seems that histopathology does not evaluate precisely the N status in some patients [15]. It has been recently shown that by slicing the lymph nodes into 2 mm tissue blocks and preparing one or more levels of hematoxylin-eosin (HE) staining from each block, as recommended by histopathological guidelines, metastases smaller than 2 mm might be missed [16]. Serious histopathology is more sensitive than conventional histopathology but it requires more intensive labor because a large number of histopathological specimens from each patient should be evaluated. That is why it is difficult to use it as a standard procedure. Immunohistochemistry is routinely used only in medullary thyroid cancer. In differentiated thyroid cancer, it is not so helpful. Some molecular examinations used for the assessment of lymph node status have shown higher positivity rates when compared to routine HE staining as well as immunohistochemistry. Nowadays lymph node metastases are diagnosed more and more often by molecular assays and immunohistochemistry [17-25]. The data of these studies may help to find more sensitive methods for the early detection of cell dissemination and may refine risk groups that might benefit from more extensive surgery or adjuvant therapy. These techniques are also performed in thyroid cancer. It has been proven that they could be useful for involved lymph node detection [26, 27]. In this study we would like to look for the most suitable real-time PCR marker that could be used in differentiated thyroid cancer. Cytokeratin 19, RET, galectin-3 and HBME-1 expression in PTC were higher than in benign thyroid diseases, but they are not specific markers for PTC [28, 29]. The diagnostic efficiency of CK19 for PTC was slightly better than galectin-3 [30]. Earlier studies have proven that thyroglobulin is a suitable marker for real-time PCR in the evaluation of lymph node metastases in thyroid cancer [26, 31]. In this study, the objective was to choose the best marker for molecular evaluation of lymph node metastases of PTC. To do this, real-time PCR examinations for TG and CK19 mRNA were performed and the obtained results were compared with histopathology as a gold standard.

## Material and methods

Total thyroidectomy with appropriate lymphadenectomy was performed on the patients included in the study. The operations were performed according to the guidelines of the Polish Society of Surgeons and the Polish Society of Oncological Surgery [32].

The studied group covered 107 selected lymph nodes, randomized from 34 patients with papillary thyroid cancer. Histopathology examination of resected tumors revealed the classic subtype of papillary thyroid cancer to be most prevalent (32 patients), with two patients with oxyphilic subtype of PTC (6 nodes). The number of evaluated nodes from each individual ranged from 1 to 8. The diameter of lymph nodes ranged from 7 to 13 mm.

In all included lymph nodes TG and CK19 expression levels were measured and histopathological examination was performed.

To obtain representative material from all lymph nodes, they were divided into four blocks by a sterile, single use, special cutting device, primarily designed for one-step nucleic acid amplification (OSNA) examination [33].

### Quantitative real-time PCR procedure

The reverse transcription reaction was performed using the commercially available set of High Capacity cDNA Archive Kit (Applied Biosystems, USA). cDNA was prepared from 1 $\mu$g of mRNA isolated using the phenol-chloroform method (Tri Reagent, Sigma-Aldrich, St. Louis, MO, USA). For the reverse transcription reaction, random hexamer primers were used, according to the manufacturer's instructions – 10 min 25°C, 2 h 37°C, and 4°C thereafter. The resulting cDNA was diluted to a final concentration of 5 ng/$\mu$l and was used as a matrix in further experiments. The analysis of TG and KRT gene expression levels was performed using a human commercial available assay Hs00794359_m1 and Hs00761767_s1 (Applied Biosystems, Foster City, CA, USA), referenced with GAPDH expression level. The analysis was carried out in the genetic analyzer 7900HT Real-Time PCR (Applied Biosystems, Foster City, CA, USA). Comparative analyses of each of these genes in individual patients were performed using specialized computer programs SDS2.3 and RQ 2.1 (Applied Biosystems, Foster City, CA, USA). All amplification reactions were performed in duplicate. The mRNA expression levels of TG and KRT were calculated using the $2^{-\Delta\Delta CT}$ (comparative threshold $C_T$ 0.09) method, as detailed by the manufacturer.

## Results

### Histopathology

Metastases were detected in 20 nodes from 10 (29.4%) patients. All of them were diagnosed with the classical subtype of PTC (Table I).

**Table I.** Results of histopathology and quantitative real-time PCR

| Lymph nodes | Histopathology | Real-time PCR TG mRNA | Real-time PCR CK19 mRNA | N |
|---|---|---|---|---|
| | negative | negative | negative | 79 |
| | negative | negative | positive | 0 |
| | negative | positive | negative | 8 |
| | negative | positive | positive | 0 |
| | positive | negative | negative | 4 |
| | positive | negative | positive | 2 |
| | positive | positive | negative | 9 |
| | positive | positive | positive | 5 |
| | total | | | 107 |

*Cut-off values with the best diagnostic efficacies were established at ddCt lower than 3.245 for TG and ddCt lower than 4.712 for CK19.*
*CK19 – cytokeratin 19, TG – thyroglobulin*

**Table II.** Diagnostic efficacy parameters of thyroglobulin and CK19 in detecting node involvement in patients with papillary thyroid cancer

| | TG < 3.245 | CK19 < 4.712 |
|---|---|---|
| Accuracy | 88% | 86% |
| Sensitivity | 78% | 35% |
| Specificity | 91% | 100% |
| Positive predictive value | 69% | 100% |
| Negative predictive value | 94% | 85% |

*CK19 – cytokeratin 19, TG – thyroglobulin*

## Thyroglobulin and cytokeratin 19 expression results

Thyroglobulin and CK19 expression levels differed vastly between nodes with and without metastatic cells. ddCt of TG in the genetic material extracted from N0 nodes was 9.97 ±4.20, while in nodes with metastases ddCt was 0.91 ±4.20 (p < 0.0001). CK19 showed similar results with expression level (ddCt) in N0 nodes of 10.96 ±2.58 vs. 7.73 ±3.63 in nodes with metastases (p < 0.0001). Levels of CK19 and TG were strongly correlated with each other (R = 0.48; p < 0.0001), although the correlation was evident in samples without metastases (R = 0.47; p < 0.0001) and absent in samples with metastases (R = 0.10; p = 0.6623), suggesting the existence of distinct molecular subtypes.

Evaluation of the diagnostic utility of both parameters showed efficient differentiation of node involvement in the case of TG, with area under the ROC curve equal to 0.91 (95% CI: 0.85-0.96). Cytokeratin 19 also allowed for a degree of differentiation but its diagnostic efficacy was lower (AUC 0.76 95% CI: 0.64-0.88). Cut-off values with the best diagnostic efficacies were established at ddCt lower than 3.245 for TG and ddCt lower than 4.712 for CK19. Diagnostic efficacy parameters are shown in Table II.

The difference between the ROC curves was statistically significant in favor of TG (p = 0.006). Cy-tokeratin 19 exhibited potential for a highly specific diagnostic test, as all nodes (N = 8) with ddCt < 4.71 contained metastatic cells. TG on the other hand had a higher detection rate of cancer (18 out of 23), but misclassified nodes as both false positive (N = 8) and false negative results (N = 5). The ROC curves for TG and CK19 are shown in Fig. 2.

Exclusion of nodes from patients with histopathological diagnosis other than the classic papillary tumor resulted in a marginal improvement of CK19 efficiency (AUC 0.81 95% CI: 0.68-0.93) and no change in TG performance (AUC 0.90, 95% CI: 0.84-0.96). Optimum diagnostic thresholds were however the same as in the whole sample.

## Discussion

Lymph node status is the most important prognostic factor in resectable thyroid cancer. Patients with N0 status have much more favorable survival rates. However, many of them have relapse, despite surgical resection. This could be plausibly explained by an inadequate lymphadenectomy or inadequate histopathological examination. It seems that histopathology, eventually with immunohistochemistry, does not precisely evaluate the TNM status. In this study we tried to estimate lymph node status using quantitative real-time PCR. We hope that in the future molecular methods can better predict prognosis in patients with solid cancers. For example, in colorectal cancer, high risk of disease recurrence and poor survival in histopathological N0 lymph nodes was indicated based on molecular methods [34].

These patients could benefit from more radical treatment. In our study, concerning thyroid cancer, we wanted to compare two molecular examinations (real-time PCR for CK19 and TG) with each other as well as the standard histopathology. We chose TG because it seems to be one of the most specific substances for the thyroid. It is found only in normal thy-
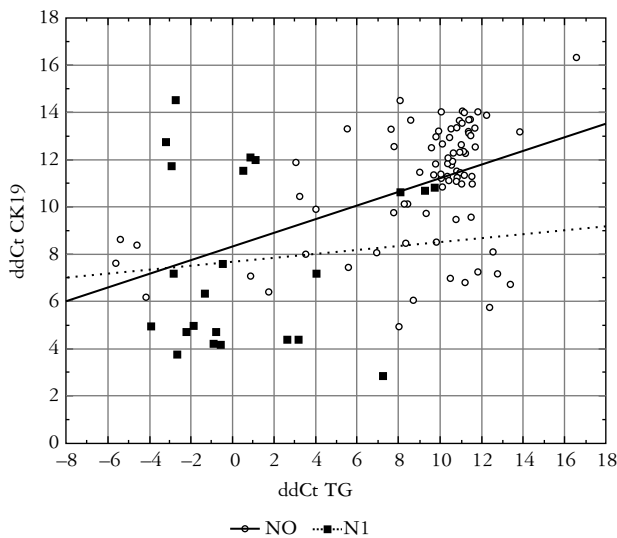
*CK19 – cytokeratin 19, TG – thyroglobulin*

**Fig. 1.** Heterogeneity of CK19 and TG expression levels depending on nodal involvement
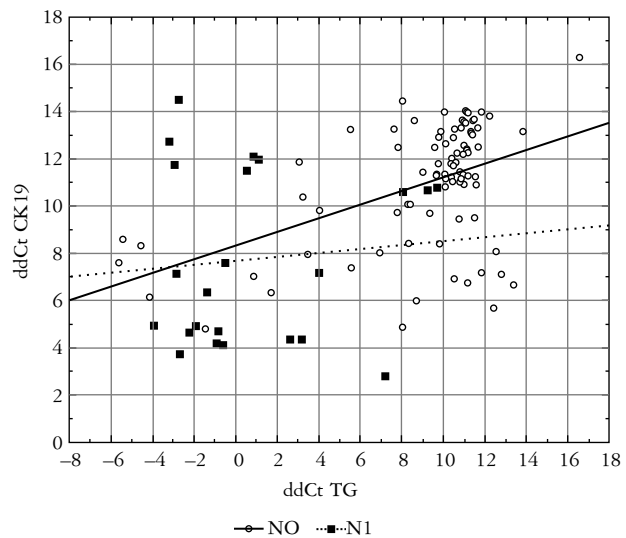


*CK19 – cytokeratin 19, TG – thyroglobulin*

**Fig. 2.** ROC curves of TG and CK19 expression levels in lymph nodes suspected of harboring thyroid cancer metastases

roid gland and cells of differentiated thyroid cancer, derived from follicular cells. Other markers, except thyroid peroxidase and NIS, are not so characteristic for thyroid gland [35].

Various low and high molecular weight cytokeratins, in general, are expressed differentially in papillary thyroid cancer [36, 37]. In particular, CK19 is demonstrated to be limited to papillary thyroid cancer, thus favoring the diagnosis of PTC in all its variant patterns [38].

Most of the previous studies have proposed CK19 as a useful marker that could be used to distinguish papillary thyroid cancer from other benign and malignant follicular lesions [39, 40]. In our study, we compared the results of histopathology with real-time PCR for CK19 and TG mRNA. According to our results if the preferred scenario would minimize node recurrence, TG could be used for screening using quantitative methods to select patients who should undergo lymphadenectomy due to an increased risk of node involvement. If reoperation can be performed safely, CK19 can be used for screening, selecting patients who are certain to show node involvement, with the rest requiring reevaluation after the histopathology workup. In the presented study TG showed a sensitivity of 88%, suggesting potential for intraoperative screening if the molecular evaluation could be performed rapidly. An interesting observation was also made when the number of individual patients was analyzed. Out of the 5 missed cases of nodular involvement, 3 samples were collected from a single patient and had low expression of both CK19 and TG (a cluster of three nodes with positive results is clearly seen in the upper right quarter of figure 2, suggesting a distinct molecular subtype

in that patient. Moreover, this patient would be missed by both the TG and CK19 thresholds, indicating that molecular heterogeneity of thyroid cancers may pose unexpected challenges to expression-based screening for nodular metastases. False positive results suggested by molecular evaluation also deserve an explanation. Eight nodes with TG expression may in fact represent a group of nodes in which no evidence of metastases was found in histopathology due to the small number of present neoplastic cells picked up by expression level evaluation in real-time-PCR. If the latter scenario is true, molecular examinations could be used to select a previously missed group of patients with nodal involvement undetectable by standard histopathological examination. Follow-up examination of these putative false positive cases will show whether molecular or histopathological evaluation was clinically more important. Complex classification created using multivariate polynomial regression of combined expression scores of both CK19 and TG allowed us to increase the AUC to 0.95 and reduce the number of false positives to 4. However, such classifiers would require additional computational steps and expression analysis of both genes doubles the workload. Our study has shown that quantitative real-time PCR for both markers TG and CK19, used in this study, is a fast, sensitive method of gene expression analysis. The application of validated probes reduces the risk of cross-reactivity. The method requires far less biological material than expression microarray analysis, uses fewer pre-analytic steps and is considerably cheaper. These qualities make it a feasible method for use in intraoperative examinations, provided that the hospital has rapid access to a genetic analyzer on standby.

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## Address for correspondence

**Krzysztof Kaczka**
Department of General and Oncological Surgery
Medical University of Lodz
Maria Sklodowska-Curie Memorial Hospital
Parzęczewska 35
95-100 Zgierz, Poland
tel. +48 503 068 786
e-mail: krzysztofkaczka@poczta.fm