Nodal status in the papillary thyroid vancer. Comparison of the results of routine histopathological examination, immunohistochemistry and reverse transcription – polymerase chain reaction.

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Immunohistochemistry (IE) and polymerase chain reaction (PCR) are tools enabling to find small number of tumor cells in lymph nodes (LNs) or peripheral blood. Perhaps these methods will allow early detection of cell dissemination and refine risk group within papillary thyroid cancer (PTC) that might benefit from more extensive surgical procedures or adjuvant therapy. In our study we detected PTC cells in the cervical LNs by routine histopathological examination RHE), IE and RT-PCR and compared obtained results. We also estimated the impact of RT-PCR and IE results on TNM staging and clinical staging according to UICC in patients with PTC. Each of 216 LNs from 28 patients with PTC were divided into two parts: one for RHE and IE the other one for Tg mRNA RT-PCR. Nodal metastases of PTC, in the regional LNs, were found by RT-PCR only in 1(3.6%) patient more than in RHE. In other 4(14.3%) patients molecular examination increased number of involved LNs. In the other patient it revealed less metastasized LNs. The molecular examination changed nodal status in 5(17.9%) of 28 patients. TNM staging was altered from N_0 to N_1 in one patient. In the others was changed only the number of involved LNs

Our research prooved that Tg mRNA RT-PCR technique was sensitive method for detection of nodal metastases of PTC. The outcomes of RT-PCR are similar to RHE so that examination really does not change the estimation of the disease staging according to UICC classification and main surgical therapy in PTC patients.

Key words: Thyroid cancer, lymph node metastases,histopathological examination immunohistochemistry, reverse transcription-polymerase chain reaction.

In spite of good prognosis, the PTC metastasizes to the regional lymph nodes (RLNs) from 11% [1] up to 90% [2] patients and gives distant metastases to 14% [3] patients. The clinical value of involved RLNs is matter of controversy. Some authors think, that it is worse prognostic factor, which decreases survival rate [4, 5] The others suggest that, the presence of metastases in the regional LNs has no clinical value [6–8].

Also the range of the lymphadenectomy is considered. Nowadays, there are two main kinds of the lymphadenectomy in the case of the PTC: modified, radical lymph node excision and selective lymphadenectomy [6–10]. We suppose that the extent of lymph node dissection is controversial because of the lack of reliable diagnostic methods for nodal metastases, others than RHE. Now the detection of disseminated tumor cells is one of the main topics in current oncology. The first immunohistochemical examinations (IE) were used in patients with neuroblastoma, lung cancer and breast cancer [11–13]. They allowed detecting neoplastic cells present among 10^4 to 10^5 other cells. Although the IE can improve detecting of neoplastic cells, they are not used so common. Some low-differentiated cancers loss expression of specific antigens used in immunohistochemical diagnosis [14]. Also many antigens are not specific for one organ or neoplasm because one type of the tissue can be found in many organs and neoplasms derived from them. For example, antigen CK-20 is present in the epithelium of digestive tube, urinary tracts, pancreas, thyroid, ovary and also neoplasm derived from these organs [15–18].

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In 1983 Kary Mullis introduced new technique- polymerase chain reaction which allows to obtain millions copies of DNA with required sequences [19]. Using proper nucleic acid and primers there is possibility to find single neoplastic cell among millions of other cells so residual disease can be detected even in cases of negative histopathological examination from bone marrow. In solid tumors cancer cells and micrometastases are found by reverse transcriptase- polymerase chain reaction (RT-PCR). In this technique cDNA, obtained on the ground of proper mRNA, is amplified [19, 20]. The further steps of the reaction are the same like in typical PCR. RT-PCR was used to detect neoplastic cells of many tumors: colorectal cancer [16, 17, 21], gastric cancer [22, 23], breast cancer [24–26], melanoma malignum [27, 28], and others [11, 29]. This procedure revealed micrometastases also in regional LNs, which are negative in histopathology.

In thyroid cancer, relapse is one of the worst prognostic factors [15, 30, 31]. It can appear most often in regional LNs, also in the cases when the RHE from removed lymph node was negative [32]. There is an urgent need to look for new techniques, which could allow better estimate lymph nodes' status in thyroid cancer patients than histopathology. Apart from our preliminary report there is only one study, in which results of histopathology of removed lymph nodes are compared with results of molecular examination [33]. It concerns medullary thyroid cancer [15]. This fact encouraged us to perform such study for papillary thyroid cancer. In our investigation we chose as marker thyroglobulin for immunohistochemistry and Tg mRNA for RT-PCR. It is one of the most specific thyroid antigens, normally presents only in thyroid cells and neoplastic cells derived from follicular cells, especially in PTC. Others, besides thyroid peroxidase and iodine symporter, are not so specific for thyroid.

The aim of the presented study was 1) detection of PTC cells in the regional LNs by RHE, IE, and RT-PCR and evaluation of the obtained results and. 2) estimation of impact of molecular and immunohistochemical examination results on TNM staging and clinical staging according to UICC in PTC patients.

Patients and methods

Twenty-eight patients with PTC treated at the Department of Endocrinological and General Surgery, the Medical University of Lodz, Poland (23 women, 5 men) were included into study (Table 1)

During the operation total thyroidectomy with central lymph node dissection (group 1, 2, according to WHO classification [35], level and VI according to Robbins' classification [36, 37], compartment according to Dralle et al. classification [38] and uni- or bilateral modified, radical neck dissection (group 3-6 according to WHO classification, level II-V according to Robbins' classification, compartment II and III according to Dralle et al. classification) was performed. Type of the operation was conformable with the guidelines of the Polish Society of Surgeons and the Polish Society of Oncological Surgery [39](Table 2).

Immediately, the removed lymph node samples were carried to the Department of Pathology and divided into two halves. One half of the each lymph node was used for RHE and IE; the other one was snap-frozen in liquid nitrogen, stored at 70°C and used for RT-PCR. To avoid contamination, the lymph nodes were cut free from other tissue and fresh, sterile scalpel was used each time. In our study, we analyzed 216 lymph nodes (from to 15 per patient, mean 7.7). The diameter of examined lymph node was from 0.3 to 2.6 cm. Smaller ones were used only for histopathology because of technical reasons. Like others we think, that it is difficult to find, in fresh tissue samples and afterwards, precisely cut lymph nodes that are smaller than 3mm [15, 16, 17].

Histopatological and immunohistochemical examination The lymph nodes were fixed in formalin, embedded in paraffin and sectioned for histological studies. The sections were stained with hematoxylin and eosin and observed under light

Table 1. Patients included into the study (M- male, F- female)

Patient	Age years)	Sex
1	34	M
2	30	F
3	51	F
4	62	M
5	60	F
6	57	M
7	44	M
8	36	F
10	58	F
11	60	F
12	51	M
13	33	F
14	44	F
15	47	F
16	19	F
17	53	F
18	48	F
19	49	F
20	61	F
21	68	F
22	56	F
23	57	F
24	23	F
25	28	F
26	20	F
27	41	F
28	57	F

Table 2. Extent of the tumor/tumors according to TNM classification from 2002.

T staging	Number of patients	%
T_{1a}	15	53.6
T_{1b}	2	7.1
T_{2a}	8	28.6
T_{2b}	1	3.6
T_{3a}	2	7.1

microscope. Immunohistochemical staining for Tg was performed on formalin-fixed, paraffin-embedded sections with anti-Tg antibodies (Dako, Germany). Presence of Tg was visualized with alkaline phosphatase.

Total RNA isolation and reverse transcriptase-polymerase chain reaction. Total cellular RNA was isolated from frozen tissue samples using commercial kit (A&A Biotechnology, Poland) according to the manufacturer's protocol. Approximately 1-2 µg of total RNA was reverse transcribed into single-strand cDNA in final volume of 20 µl containing µl x buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 1.5 µg oligo(dT), 0.5 mM dNTP, 20 units Rnase inhibitor (Promega, Madison, WI, USA) and 200 units M-MLV reverse transcriptase (Promega). First-strand cDNA synthesis reaction was performed at 37°C for 90 min. The following PCR reactions were performed in final volume of 20 µl containing ml of the RT-solution, µl 10 reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl), 0.5 µM of each primer, 0.2 µM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 units Taq-DNA polymerase (Promega). 348-bp fragment of transcript of TG gene and 548-bp fragment of transcript of β-actin gene were amplified in separate PCR reactions using the following primers: TG forward 5'-TGTGAGCTGCAGAGGGAAACGGCC-3'

TG reverse 5'-TGTGTGGACGCAGAGGGGATGGAGGT GTAT-3'

β-actin forward 5'- GTGGGGCGCCCCAGGCACCA-3';

β-actin reverse 5'-CTCCTTAATGTCACGCACGATTTC-3'.

After denaturation for min. at 94 °C the total amount of reaction products was amplified for 35 cycles for TG (94 °C, 30 sec; 56 °C, 30 sec; 72 °C, 60 sec) and 35 cycles for b-actin (94 °C, 30 sec; 58 °C, 30 sec; 72 °C, 30 sec) on the Pelitier Thermal Cycler system (PTC-200, MJ Research). The PCR products were resolved on 2% agarose gel and visualized by ethidium bromide staining on UV table.

In each experiment water was used as negative control and sample with presence of Tg RNA as positive control. The results of molecular studies were presented after computer image analysis using the system Gel Doc1000 Bio Rad Image with the program Molecular Analyst applied.

Results

Histopathological examination of the removed LNs. Localization of the lymph nodes was defined according to Dralle et al. classification [38].In RHE nodal metastases were found in 33 lymph nodes of 91 LNs (12 cervicocentral compartment, 12 right lateral compartment, left lateral compartment) from 11 patients. In 17 patients there were no metastases to regional lymph nodes according to histopathology. (Table 3)

One hundred percentage correspondence was observed between RHE and IE so we analyzed these results as one group (pathological examination). In all cases IE confirmed positive results of the RHE from lymph nodes.

Molecular examination. We obtained positive results of Tg mRNA RT-PCR in 41 lymph nodes (14 right lateral compartment, 12 left lateral compartment and 15 central compartment) from 12 patients (table 3). Only in one patient RT-PCR detected metastatic lymph node involvement where previously all the LNs were free on histopatological examination.

The molecular examination found more positive LNs, than histopathology in 3 patients and one more in one patient In the other patient, the molecular examination was less sensitive than histopathology. By the histopathology that patient had metastasized LNs (right lateral compartment), Tg mRNA was revealed in LNs from that compartment. However, RT-PCR did not changed TNM staging in these patients.

Discussion

Histopathology, with additional methods such as immunohistochemistry, is the main method of examination of RLNs in cancer cases. It seems however that it does not evaluate TNM

Table 3. Nodal metastases in the regional lymph nodes according to the pathological and molecular examinations- comparison

Patient	The involved nodes examined by immunohistochemistry					The involved lymph Nodes examined by RT-PCR				
	Compartment				Total	Compartment				Total
	I	II	III	IV		I	II	III	IV	
4	4	1	0	0	5	4	1	0	0	5
6	0	3	0	0	3	0	5	0	0	5
9	0	0	0	0	0	2	0	0	0	2
11	0	0	3	0	3	0	0	3	0	3
13	5	0	0	0	5	5	0	0	0	5
16	0	0	3	0	3	0	0	5	0	5
19	0	3	0	0	3	0	2	0	0	2
21	0	3	0	0	3	0	3	0	0	3
23	0	1	0	0	1	1	2	0	0	3
25	0	0	2	0	2	0	0	2	0	2
26	0	0	1	0	1	0	0	2	0	2
28	3	1	0	0	4	3	1	0	0	4
Total	12	12	9	0	33	15	14	12	0	41

staging in some patients precisely. Locoregional relapse may be also present even if the RLNs are free in RHE. Perhaps, it is caused by limited lymphadenectomy and/or micrometastases not detected in RHE. Finding single or small clusters of neoplasmatic cells in slides may be difficult for pathologist. There is only 1% probability of detection of the micrometastases having less than 3 cells [40, 41]. The RHE has probably limited value because only 1 or 2 slides from each biological material are examined. [42]. Gusterson et al., using serial histopathological examination (SHE), found micrometastases in about 20% colorectal patients with not involved lymph nodes in RHE [43]. Nahrig et al and Pargaonkar et al proved the higher sensitivity of SHE examination in searching of sentinel lymph nodes in breast cancer patients [44]. Both groups of the authors detected more involved lymph nodes by that examination. The SHE has also one fault. It requires to make much more histopathological specimens from each patient so it is difficult to use it as standard procedure.

In RHE, without using monoclonal antibodies, there is no possibility to determine antigens present on cells. More reliable defining tissue origin of examined material can be reached by immunohistochemistry, which should be standard in some cases. This examination proved its utility in diagnosis of material obtained during FNAB from thyroid gland or pathologic masses of the neck.

The studies comparing histopathology with IE and molecular examinations in detection of the metastasized lymph nodes were performed in many neoplasms such as: colorectal cancer [45, 46], breast cancer [47] oesophageal cancer [48] melanoma malignum [49]. We have not found such study for the differentiated thyroid cancer except our preliminary report [33]. Only Weber et al. made such comparison for the medullary thyroid cancer [15]. To find nodal metastases in IE and RT-PCR they used the same marker- CK20. In 31 (74%) of 42 lymph nodes the same results were obtained. In (19%) lymph nodes RT-PCR found micrometastases. According to RHE and IE they were free from neoplasmatic cells. Positive IE results were also obtained from (7%) lymph nodes negative in RT-PCR.

In our study we decided to perform such examination for the removed lymph nodes of the PTC. We chose thyroglobulin as marker. It seems one of the most specific substances for thyroid because it is present only in normal thyroid gland and cells of the differentiated thyroid cancer, derived from the follicular cells. Others, except thyroid peroxidase and NIS, are not so characteristic for thyroid gland [50]. In addition, searching for thyroglobulin in IE and mRNA for thyroglobulin in the same lymph node we avoided making mistake caused by different expression of various markers.

In our research 100% correspondence was observed between RHE and IE. This result confirms high specificity of IE and good performing of RHE. In both examinations nodal metastases were found in 33 lymph nodes from 11 (39.3%) among 28 patients (Table 3).

In four patients (1. 8. 12. 14. Table1) positive results of IE were in several lymph nodes. There were lymph nodes from patients (1, 8) and lymph node from other patients (12, 14).

Our pathologists described the presence of thyroglobulin in macrophages from these lymph nodes but they could not find neoplasmatic cells. Like Venkatraman et al. we think, these lymph nodes are free of cancer cells [51]. It is interesting that these lymph nodes were also negative in RT-PCR.

The presence of involved RLNs, detected by pathological methods, in 39.3% patients is not very high but comparable to other authors [38]. Among our patients 53.6% had T_{1a} tumor (table 2.) and none had T_4 tumor according to the TNM classification from 2002. Perhaps there are the reasons of finding nodal metastases only in 39.3% of the patients.

Cells of the PTC in the RLNs preserve ability to express specific genes so it seems that detection of this expression is synonymous with finding nodal metastases in RHE. Like in the morphological examinations 216 lymph nodes were searched by RT-PCR.

In 22 (78.6%) patients there were the same results of three examinations. Sixteen (57.1%) people had no metastasized lymph nodes. In (21.4%) ones, the nodal metastases were found in the same lymph nodes (Tables 3).

Tg amplification products were revealed in 41 RLNs from 12 (42.9%) patients The molecular examination detected two more positive lymph nodes, than RHE in the three patients (6. 16. 23.) and one more in one patient (26.HD) (Table 3).

In RT-PCR patient 9.KF had two metastasized lymph nodes in the cervicocentral compartment (table 3.). The morphological examination detected no involved lymph nodes in this patient.

In our study RT-PCR detected, more than histopathology, involved lymph nodes in 5(17.9%) patients but only in 1(3.6%) patient it has changed TNM staging, but not clinical staging according to UICC (34). In our study RT-PCR results have not such great impact on staging evaluation as in other cancers [21, 27, 28].

Introducing so sensitive method as RT-PCR, in diagnosis of removed lymph nodes makes problem with interpretation of its results. Is it sure that positive RT-PCR result is synonymous with the presence of nodal metastases? Extremely high sensitivity, the main advantage of RT-PCR, is also its fault. The more cycle of PCR is performed, the false positive results is more possible to obtain. It may be caused by illegitimate amplification or by pseudogenes [20, 52–55]. Phenomenon of illegitimate transcription in case of the PTC was described by Bojunga et al. [56] and Austin et al. [55]. They proved that increasing, number of the PCR cycle for Tg mRNA to 40, causes losing its specificity. Therefore in our study we performed only 30 cycles of PCR. We hope, that in this way we did not observe false positive results. In the patient (19.SM) the molecular examination was less sensitive than histopathology. According to the RHE this patient had three metastasized lymph nodes in the right cervicolateral compartment (Table 3.). Tg mRNA was revealed in lymph nodes from the same compartment. At the beginning this result was explained by degradation of RNA during transport or

preservation. However we observed genetic material in β-actin control from repeated isolation. The similar problem had the group of German researchers [15]. Like they we suppose that the cancer involved only one part of the lymph node. The other one, free from neoplasmatic cells was used for the molecular examination. Due to of lack of material to perform RT-PCR from two parts of lymph node it is impossible to verify this hypothesis. It is fault of the method but there is no procedure to avoid this limitation.

Based on the gained results we can conclude that Tg mRNA RT-PCR technique is sensitive method in detection of nodal metastases of the PTC. However it is interesting that the outcomes of RHE and molecular examination are similar. Nowadays, the molecular examination really does not influence the clinical staging in UICC classification and the extent of surgical therapy in PTC patients. This result is different from the studies in cases of other cancers, in which the RLNs were examined also by similar molecular methods [21, 27, 28]. It encourages to perform further studies on PCR examination in PTC. We hope it will be possible to improve its specificity and sensitivity for better assessment of PTC patients. From the surgical point of view, it is very important to perform intra-operative examination to detect the lymph nodes metastases. Now this diagnosis can be performed by staining of frozen sections. However, this method is not standardised and does not detect all metastases since only part of the lymph node is examined. Our examination proved that RT-PCR is as accurate as the conventional method but the analysis takes several hours. To address that problem we try to establish new intra-operative molecular diagnostic tool for PTC lymph node metastases. Such examination was performed for breast cancer patients by Tsujimoto et al. [57]. We hope it is also possible for PTC patients.

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