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First one-step nucleic acid amplification testing in papillary thyroid cancer lymph nodes — a comparison with histopathology and real-time PCR

Badanie metodą jednostopniowej amplifikacji kwasu nukleinowego węzłów chłonnych w raku brodawkowatym tarczycy — porównanie z badaniem histopatologicznym i badaniem PCR w czasie rzeczywistym

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Abstract

Introduction: The significance of lymph node metastases and the optimal extent of lymphadenectomy remain matters of controversy in papillary thyroid cancer.
This study was designed to assess the feasibility and reliability of OSNA and real-time PCR for CK19 and TG mRNA in papillary thyroid cancer lymph nodes evaluation compared to standard histopathology.
Material and methods: Each of 92 randomised lymph nodes from 32 papillary thyroid cancer patients were divided into representative parts and assessed using the three studied methods.
Results: Eighteen (19.6%) lymph nodes from ten (31.3%) patients were positive according to histopathology. When the cut-off value distinguishing metastatic from non-metastatic lymph nodes in the OSNA assay was set at 250 copies per microlitre, the results were positive in 16 (17.4%) lymph nodes from 11 (34.4%) patients. Twenty three (25%) lymph nodes were tested positive in real-time PCR for TG mRNA. Real-time PCR for CK19 mRNA was positive in 18 (19.6%) lymph nodes from 13 (40.6%) patients. No statistically significant differences were noted between the diagnostic accuracy of either molecular method compared to the histopathological examination ($p = 0.81$). Overall, 20 positive molecular biology results were noted in patients with negative histopathology results. Conversely, in 18 lymph nodes, despite a metastasis finding in histopathology, at least one molecular test yielded a negative result.
Conclusions: It was revealed that OSNA is a reliable technique for the evaluation of lymph node metastases in papillary thyroid cancer. This method was shown to have equivalent accuracy to histopathology and real-time PCR. **(Endokrynol Pol 2014; 65 (6): 422–430)**

Key words: *OSNA; histopathology; real-time PCR; lymph nodes; papillary thyroid cancer*

Streszczenie

Wstęp: Znaczenie przerzutów do węzłów chłonnych oraz optymalny zakres limfadenektomii w raku brodawkowatym tarczycy pozostaje przedmiotem kontrowersji.
Celem pracy była ocena wykonalności oraz zgodności wyników jednostopniowej amplifikacji kwasu nukleinowego oraz PCR w czasie rzeczywistym dla CK19 i TG mRNA w badaniu węzłów chłonnych w raku brodawkowatym tarczycy w porównaniu z rutynowym badaniem histopatologicznym.
Materiał i metody: Każdy z 92 węzłów chłonnych pochodzących od 32 pacjentów z rakiem brodawkowatym tarczycy został podzielony na reprezentatywne części i zbadany trzema metodami.
Wyniki: Osiemnaście (19,6%) węzłów chłonnych od 10 (31,3%) pacjentów miało dodatni wynik badania histopatologicznego. Przyjmując wartość odcięcia 250 kopii w mikrolitrze, różnicującej węzły chłonne zmienione przerzutowo od niezmienionych, w badaniu jednoetapowej amplifikacji kwasu nukleinowego, stwierdzono dodatni wynik badania w 16 (17,4%) węzłach chłonnych od 11 (34,4%) pacjentów. Uzyskano dodatni wynik badania PCR w czasie rzeczywistym dla TG mRNA w 23(25%) węzłach chłonnych, natomiast dla CK19 mRNA w 18 (19,6%) od 13 (40,6%) pacjentów. Nie stwierdzono istotnej statystycznie różnicy pomiędzy zgodnością wyników obu metod molekularnych z wynikiem badania histopatologicznego ($p = 0,81$). Ogólnie stwierdzono 20 dodatnich wyników badania molekularnego z węzłów chłonnych, przy ujemnym wyniku badania histopatologicznego. Natomiast w 18 węzłach, pomimo znalezienia przerzutów w badaniu histopatologicznym, uzyskano ujemny wynik w przynajmniej jednym badaniu molekularnym.
Wnioski: Badanie jednostopniową amplifikacją kwasu nukleinowego jest właściwą metodą oceny obecności przerzutów w węzłach chłonnych w raku brodawkowatym tarczycy. Technika ta ma zbliżona wiarygodność do badania histopatologicznego i badania PCR w czasie rzeczywistym. **(Endokrynol Pol 2014; 65 (6): 422–430)**

Słowa kluczowe: *OSNA; histopatologia; badanie PCR w czasie rzeczywistym; węzły chłonne; rak brodawkowaty tarczycy*

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Introduction

Patients with papillary thyroid cancer (PTC) have a high incidence of lymph node metastases. This varies from 11% to 90% of patients at the time of surgery or histological examination [1–3]. The proper treatment and significance of cervical lymph node metastases in PTC is controversial. It has been shown in some publications that, in subsets of patients, especially younger than 45, lymph node metastases do not decrease the survival rate [4–6]. Other publications, on the contrary, suggest that lymph node involvement is a significant prognostic factor that increases the risk of locoregional relapse [7] and decreases the survival rate [8, 9]. The optimal extent of lymph node dissection also remains a matter of debate in PTC. It ranges from selective lymphadenectomy to a formal comprehensive neck dissection (prophylactic lymphadenectomy). Prophylactic lymphadenectomy has not yet been accepted by all as a standard management of occult metastases, due to the inherent risks of hypocalcaemia and recurrent laryngeal nerve injury [10]. On the contrary, according to others, selective lymphadenectomy is not sufficient and is associated with a greater risk of local recurrence [7, 9, 11]. Recurrence can appear also in patients when the routine histopathology from removed lymph nodes has been negative [12]. The significance of lymph node metastases and the extent of lymph node dissection is a matter of controversy, probably because of a lack of suitable diagnostic tools. Today, the clinical staging according to the UICC classification depends on routine histopathological examination, which eventually is allied with immunohistochemistry. In other cancers, the situation is different [13, 14]. We hope that introducing molecular methods will improve the assessment of PTC. The reverse-transcription polymerase chain reaction (RT-PCR) is used to analyse tumour-specific mRNA. As a molecular biology-based technique, it has been shown to be useful in detecting lymph node metastases. This method shows a higher sensitivity for revealing minor tumour deposits in lymph nodes compared to conventional histopathology [15–17]. However, this method is not yet used in routine clinical practice, due to its complexity and time-consuming nature. Moreover, it cannot be used as an intraoperative examination. The sentinel lymph node biopsy (SLNB) has been introduced to limit the extent of lymphadenectomy in PTC patients [18–20]. There are several advantages of the SLNB in clinical practice. It may identify patients who can benefit from lymph node dissection in the central and lateral compartment of the neck. SLNB finds lymph nodes which are at a high risk of metastases. These lymph nodes should be evaluated carefully. An intensive examination of the rest of the cervical lymph nodes may be impractical and expensive. Another important benefit of SLNB is that it can detect involved lymph nodes in the lateral compartment of the neck in patients with unaffected lymph nodes in the central compartment. The lateral skip metastasis rate in patients with thyroid cancer has been reported to be as high as 20%, and the rate of contralateral lymph node involvement as high as 18% [21, 22]. Despite these advantages, it is probably too early to consider the SLNB method as a standard of examination in the treatment of the PTC patients. If this technique becomes more common, it could be possible to apply the method in the intraoperative examination of lymph nodes. Applying the abovementioned methods will allow reoperation to be avoided. Additionally, it is impossible to intraoperatively examine the entire LN using frozen sections, which may lead to false-negative results. Indeed, the sensitivity of the intraoperative frozen section for finding nodal metastases within SNs during surgery has been reported to be 47% in melanoma patients and 74% in breast cancer patients [23]. The sensitivity of touch imprint cytology is comparable to that of frozen section examination [24, 25].

In order to find other methods of thyroid cancer lymph node evaluation, we tried to establish a new intra-operative molecular diagnostic tool. Currently there are two molecular techniques used for intraoperative assessment. Real-time PCR has been employed to estimate lymph nodes in PTC yet it is still in the research stage and is not yet used in clinical practice [26, 27]. To the best of our knowledge, PTC lymph nodes have not yet been evaluated by one-step nucleic acid amplification (OSNA). It has been demonstrated that CK19 can be used as a marker of PTC in immunohistochemical and molecular examinations [8, 28–31]. If the sentinel lymph node biopsy is introduced to thyroid surgery, OSNA could be a reasonable alternative to 'classical' intraoperative examinations. Probably OSNA could replace frozen section examination of lymph nodes in differentiating between benign and malignant follicular-type of the thyroid gland.

This study was designed to assess the feasibility and reliability of OSNA and real-time PCR for CK19 and TG mRNA in PTC lymph nodes evaluation compared to standard histopathology.

Material and methods

The study was performed in concordance with the outlines of the Ethical Commission of the Medical University of Łódź and in compliance with the Declaration of Helsinki. Real-time PCR and OSNA did not affect the operation procedure.

Characteristics of patients

A prospectively recruited cohort of 32 consecutive patients with thyroid cancer underwent total thyroidectomy with appropriate lymphadenectomy at the Department of Gen-

eral and Oncological Surgery of the Medical University of Łódź, Poland from 1 April, 2011 to 1 March, 2013. They were suspected or diagnosed with thyroid carcinoma based on FNAB. The patients were primarily operated. Patients admitted to the Department for completion thyroidectomy or removing relapse were excluded from the study. The type of operation was consistent with the guidelines of the Polish Society of Surgeons and the Polish Society of Oncological Surgery [32]. The study group consisted of seven (21.9%) men and 25 (78.1%) women with a mean age of 46.7. The patients were diagnosed with the classical subtype of PTC N = 29 (90.6%), follicular subtype of PTC N = 2 (6.3%), and oxyphilic subtype of PTC N = 1 (3.1%) (Table I). None of the patients had any significant comorbidities. Two patients were not included for the study because informed consent was not obtained.

Table I. *Histological subtype and number of examined nodes*
Tabela I. *Podtyp histologiczny i liczba zbadanych węzłów chłonnych*

Patient	Histopathology	N nodes
1	Classical subtype of PTC	3
2	Classical subtype of PTC	1
3	Classical subtype of PTC	5
4	Classical subtype of PTC	3
5	Classical subtype of PTC	2
6	Classical subtype of PTC	5
7	Classical subtype of PTC	3
8	Classical subtype of PTC	2
9	Classical subtype of PTC	2
10	Classical subtype of PTC	2
11	Classical subtype of PTC	3
12	Classical subtype of PTC	1
13	Classical subtype of PTC	2
14	Classical subtype of PTC	3
15	Classical subtype of PTC	1
16	Classical subtype of PTC	1
17	Classical subtype of PTC	2
18	Classical subtype of PTC	3
19	Classical subtype of PTC	4
20	Classical subtype of PTC	6
21	Classical subtype of PTC	3
22	Oxypholic subtype of PTC	4
23	Classical subtype of PTC	1
24	Classical subtype of PTC	6
25	Classical subtype of PTC	3
26	Classical subtype of PTC	5
27	Classical subtype of PTC	4
28	Classical subtype of PTC	3
29	Follicular subtype of PTC	1
30	Classical subtype of PTC	3
31	Classical subtype of PTC	2
32	Follicular subtype of PTC	3

Lymph nodes samples division

The number of evaluated nodes from each individual ranged from one to six. The diameter of lymph nodes ranged from 8 mm to 13 mm. All lymph nodes were separated from fat tissue before division. Each of the 92 randomised lymph nodes was divided into representative parts and assessed using the three studied methods. Firstly, they were divided into four blocks by a sterile, single use, special cutting device in the same way as in the clinical protocol study performed by Tsujimoto et al. [33]. In our procedure, blocks a and c were used for OSNA. Blocks b and d were cut along the longitudinal axis into representative sections for histopathology and real-time PCR. We did not have enough material to perform immunohistochemistry.

Histopathology procedure

The final histological examination consisted of a detailed analysis of the lymph node tissue sections embedded in paraffin blocks. All specimens were examined by an experienced specialist of clinical pathology, using a conventional optical microscope. The pathologist who performed the histopathological evaluation was blinded to real-time PCR and OSNA results.

Real-time PCR procedure

The reverse transcription reaction was performed using the commercially available set of High Capacity cDNA Archive Kit (Applied Biosystems, USA) in the manufacturer's facilities. RNA extraction was performed using 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.

Ct values of TG and CK-19 indeed showed a large variability but the dCt values before and after reverse transcription did not deviate by more than ± 15% without any biased deviation in samples positive and negative for the presence of tumour cells. This led us to believe us that RT did not introduce any significant bias and we used cDNA in further analyses.

For OSNA this step could well be omitted, accelerating the time-to-result. However, for the purpose of this study we used reverse transcription to obtain stable cDNA material for further tests of method comparisons and long-term storage.

The cDNA/RNA content in samples after isolation was measured using the spectroscopic method on the

NanoDrop device (NanoDrop, Wilmington, DE, USA). 260/280 ratios > 1.9 were considered acceptable.

The resulting cDNA was diluted to a final concentration of 5 ng/μl and used as a matrix in further experiments. Real-time PCR was performed using Taqman probes specific for TGc and CK-19 (assay numbers Hs00794359_m1 and Hs00761767_s1 (Applied Biosystems, Foster City, CA, USA), referenced with GAPDH expression level. Mean Ct of GAPDH was 25.68 ± 1.43 and was stable throughout the dataset. Comparative analyses of each of these genes in individual patients were performed using specialised computer programs SDS2.3 and RQ 2.1 (Applied Biosystems, Foster City, CA, USA). All amplification reactions were performed in duplicates. The mRNA expression levels of TG and CK-19 were calculated using the 2^{-dCt} [34]. All molecular studies were performed by an experienced molecular biologist/geneticist independently from the histopathology procedure and the results of molecular studies did not affect therapeutic decisions.

OSNA procedure

The OSNA protocol for each lymph node consisted of homogenisation of tissue in a mRNA-stabilising solution and subsequent amplification. It was made automatically by reverse transcription loop-mediated isothermal amplification (RT-LAMP) of CK19 mRNA in the RD-100i detection engine (Sysmex) without prior mRNA isolation and purification. RD-100i includes a ready-to-use reagent kit (Lynoamp, Sysmex) consisting of the enzyme, primers, nucleotides, buffer necessary for CK19 mRNA amplification and components for assay validation (calibrators, positive and negative controls). The technique uses six primers, which increase the specificity and speed of the reaction. The expression level of CK19 mRNA is detected by real-time monitoring of turbidity changes caused by an increase in the magnesium pyrophosphate concentration, a by-product of the amplification reaction. Results are automatically characterised by the CK19 mRNA copy number/LL of the original tissue homogenate in accordance with cut-off levels defined by Tsujimoto et al. [33].

The study was focused on comparing lymph nodes with or without metastases detectable in histopathology. We did not use lymph nodes from healthy individuals as additional controls due to ethical concerns, as well as due to the sufficient number of histopathology negative lymph nodes for comparison purposes.

Statistical analysis

Continuous variables were compared using Mann-Whitney's U test. Categorical variables were compared using Yates' corrected Chi^2 test or McNemar's Chi^2 test for dependent cases. Receiver operating characteristic (ROC) curves were constructed to establish optimal cut-off scores for continuous variables. Statistical 10.0 software was used for analysis (Statsoft, Tulsa, OK, USA). 95% Confidence Intervals were computed where possible. A p value lower than 0.05 was considered as statistically significant.

Results

Histopathology results

Eighteen (19.6%) lymph nodes from ten (31.3%) patients were positive. According to histopathology, no metastases were found in 63 (68.5%) regional lymph nodes coming from 22 (68.7%) patients.

OSNA results

When the cut-off value distinguishing metastatic from non-metastatic lymph nodes in the OSNA assay was set at 250 copies per microlitre, the results were positive in 16 (17.4%) lymph nodes from 11 (34.4%) patients. The results for lymph nodes in 21 (65.6%) patients were negative.

Real-time PCR

Expression of CK-19 and TG differed significantly (both p values < 0.0001) depending on the result of histopathological examination (Figures 1 and 2 respectively).

Cut-off values for real-time PCR were selected using the ROC curve analysis and they provided clinically-efficient discrimination of lymph nodes with and without metastatic cancer cells (Table II). Results of the OSNA tests were close to real-time PCR measurements of TG and CK-19, but provided respectively one and three additional erroneous measurements (Table II).

Diagnostic efficacy of OSNA measurements and real-time-PCR for TG and CK-19. 95% Confidence Interval (95% CI). Areas under the ROC curve equalled 0.82 (95% CI 0.72–0.89) for TG and 0.81 (95% CI 0.71–0.88) for CK19.

No statistically significant differences were noted between the diagnostic accuracy of either method compared to the histopathological examination (p = 0.81).

Discordance cases analysis

Clinical data

Discordant cases between histopathology and OSNA results

The results were discordant in seven (7.6%) lymph nodes. The OSNA assay was positive in three lymph nodes, while it was negative in histopathology. OSNA was not able to detect metastases in four histopathology positive lymph nodes.

Real-time PCR for TG mRNA results

23 (25%) lymph nodes were tested positive in this examination. They came from 12 (37.5%) patients. The lymph nodes in 20 (62.5%) patients were negative.

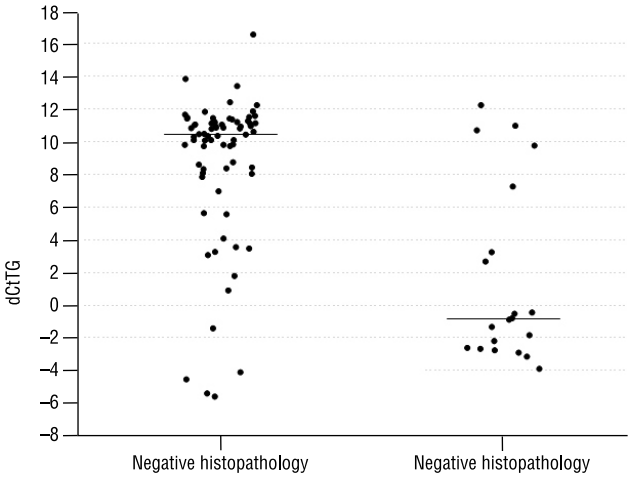

Figure 1. *Thyroglobulin (TG) mRNA expression levels in lymph nodes with and without metastatic cancer cells detected in histopathological examination*

Rycina 1. *Poziomy ekspresji mRNA dla tyreoglobuliny (TG) w węzłach chłonnych z obecnością lub bez komórek nowotworowych w badaniu histopatologicznym*

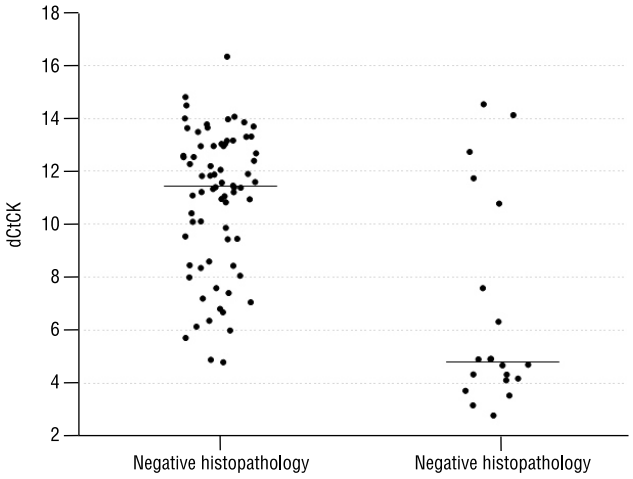

Figure 2. *Cytokeratin 19 (CK-19) mRNA expression levels in lymph nodes with and without metastatic cancer cells detected in histopathological examination*

Rycina 2. *Poziomy ekspresji mRNA dla cytokeratyny (CK-19) w węzłach chłonnych z obecnością lub bez komórek nowotworowych w badaniu histopatologicznym*

Table II. ***Diagnostic efficacy of OSNA measurements and Real-time-PCR for TG and CK-19. 95%CI — 95% Confidence Interval. Areas under the ROC curve equalled 0.82 (95% CI 0.72-0.89) for TG and 0.81 (95% CI 0.71-0.88) for CK19***

Tabela II. ***Skuteczność diagnostyczna badań OSNA i PCR w czasie rzeczywistym dla TG i CK-19. 95-procentowy przedział ufności. Pola pod krzywą ROC wynosiły 0,82 (95% CI 0,72–0,89) dla TG i 0,81(95% CI 0,71–0,88) dla CK19***

	Cutt-off	Sensitivity	Specificity	PPV	NPV
TG	< 3.25	75%	89%	65%	93%
CK-19	< 6.34	70%	93%	92%	74%
OSNA	N.A.	65%	90%	65%	90%

Discordant cases between histopathology and real-time PCR for TG mRNA results

The results were discordant in 11 (12%) lymph nodes from five (15.6%) patients. Eight (8.7%) lymph nodes were positive in real-time PCR although there were no metastases in these nodes according to histopathology. On the other hand, molecular examination was not able to detect metastases in three (3.3%) histopathology positive lymph nodes.

Real-time PCR for CK19 mRNA results

This molecular technique was positive in 18 (19.6%) lymph nodes from 13 (40.6%) patients. The other 19 (59.4%) patients had negative results in all lymph nodes.

Discordant cases between histopathology and real-time PCR for CK19 mRNA results

Discordant results between these examinations were observed in ten (9.2%) lymph nodes. Five of them were positive only in the molecular examination. On the contrary, metastases were observed only in histopathological examination in five lymph nodes. No significant bias for false positive or false negative results was detected when comparing real-time PCR for TG *vs.* OSNA (McNemar's Chi-square test p = 0.64), OSNA *vs.* real-time PCR for CK-19 (p = 1.00) and real-time PCR for TG *vs.* real-time PCR for CK-19 (p = 0.45).

Curiously false positive and false negative lymph nodes overlapped poorly between the three methods (Figs. 3–5) with ten discordant cases in all three combinations of molecular methods (OSNA *vs.* real-time PCR for TG, OSNA *vs.* real-time PCR for CK-19 and real-time PCR for CK-19 *vs.* real-time PCR for TG.

None of the lymph nodes was detected to be falsely negative by all three methods; two were negative for real-time PCR for TG and CK-19; and another two were negative for CK-19 real-time PCR and OSNA. Clustering of erroneous results was observed in OSNA and TG real-time PCR measurements (Figs. 6, 7).

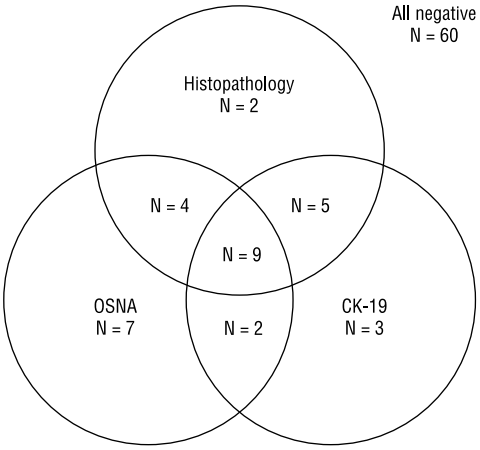

Figure 3. *Overlap of lymph nodes marked as positive in one-step nucleic acid amplification (OSNA), Real-time PCR for CK19 and histopathology. All three molecular methods and the histopathological examination yielded concordant, negative results in 60 cases*

Rycina 3. *Zgodność dodatnich wyników z węzłów chłonnych w badaniu jednoetapowej amplifikacji kwasu nukleinowego (OSNA), PCR w czasie rzeczywistym dla CK19, i histopatologii. Metody molekularne i histopatologia dały zgodne, ujemne wyniki w 60 przypadkach*

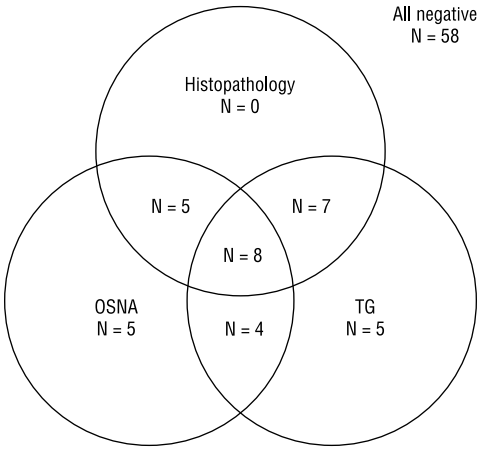

Figure 4. *Overlap of lymph nodes marked as positive in one-step nucleic acid amplification (OSNA), real-time PCR for thyroglobulin (TG) and histopathological examination. Molecular methods and the histopathological examination yielded a concordant, negative results in 58 cases*

Rycina 4. *Zgodność dodatnich wyników z węzłów chłonnych w badaniu jednoetapowej amplifikacji kwasu nukleinowego (OSNA), PCR w czasie rzeczywistym dla tyreoglobuliny (TG), i histopatologii. Metody molekularne i histopatologia dały zgodne, ujemne wyniki w 58 przypadkach*

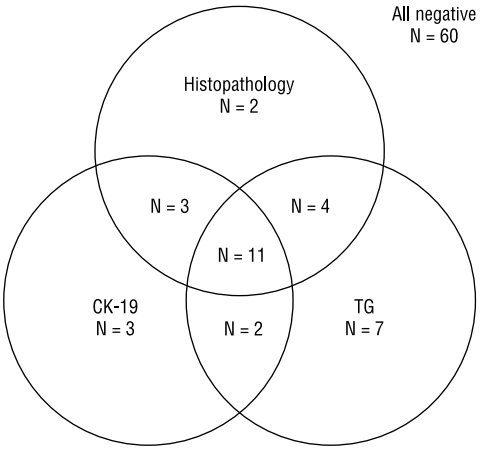

Figure 5. *Overlap of lymph nodes marked as positive in Real-time PCR for thyroglobulin (TG) or cytokeratin 19 (CK-19) and histopathological examination. Molecular methods and the histopahological examination yielded a concordant, negative result in 60 cases*

Rycina 5. *Zgodność dodatnich wyników z węzłów chłonnych w badaniu PCR w czasie rzeczywistym dla tyreoglobuliny (TG) i CK-19 (CK-19) i histopatologii. Metody molekularne i histopatologia dały zgodne, ujemne wyniki w 60 przypadkach*

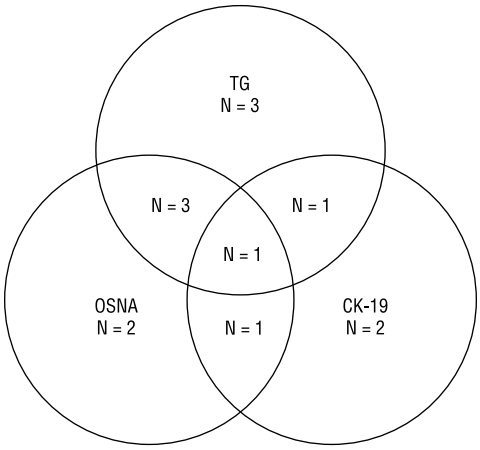

Figure 6. *Overlap of false positive results in one step nucleic acid amplification (OSNA) and thyroglobulin (TG) or cytokeratin-19 real-time-PCR (CK-19)*

Rycina 6. *Zgodność fałszywie dodatnich wyników w badaniu jednoetapowej amplifikacji kwasu nukleinowego (OSNA), w badaniu PCR w czasie rzeczywistym dla tyreoglobuliny (TG) i cytokeratyny-19 (CK-19)*

Overall, 20 positive molecular biology results were noted in patients with negative histopathology results, and were considered false positives. Those 20 results were observed in 13 individual samples, of which in five cases two methods yielded a concordant positive results despite the apparent absence of tumour cells in the histopathological examination. In one case, all three molecular methods showed evidence of tumour cells. Conversely, in 18 lymph nodes despite a positive histopathological result, at least one molecular test yielded a negative result. Of those 18 cases, four samples were termed negative by two methods and as such

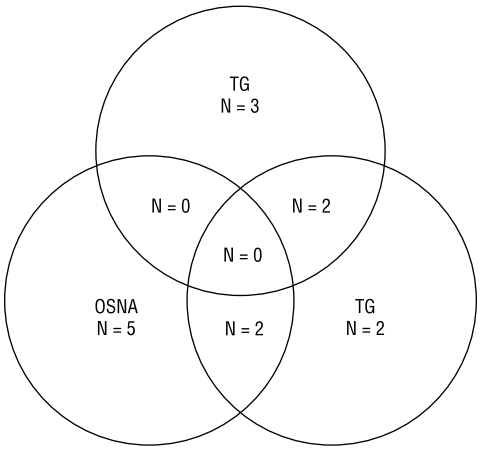

Figure 7. *Overlap of false negative results in one step nucleic acid amplification (OSNA) and thyroglobulin (TG) or cytokeratin-19 real-time-PCR (CK-19)*

Rycina 7. *Zgodność wyników fałszywie ujemnych w badaniu jednoetapowej amplifikacji kwasu nukleinowego (OSNA), w badaniu PCR w czasie rzeczywistym dla tyreoglobuliny (TG) i cytokeratyny-19 (CK-19)*

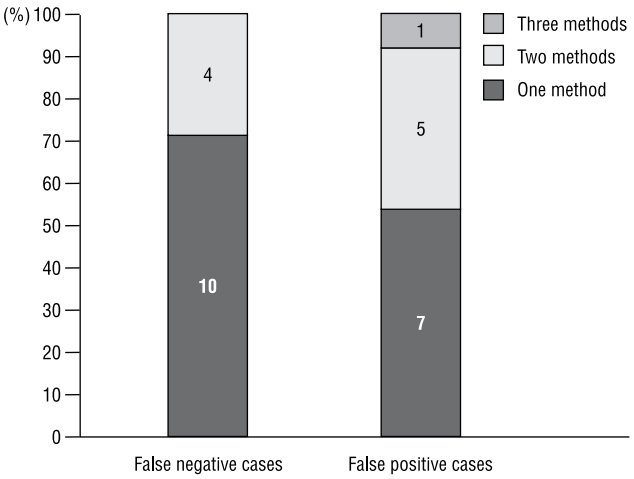

Figure 8. *Overlap of false positive and false negative results*

Rycina 8. *Zgodność wyników fałszywie dodatnich i ujemnych*

were categorised as false negative results. Therefore, it seems that a greater degree of overlap is observed in false positive cases than in false negative ones (Fig. 8), suggesting that at least some nodes deemed negative in histopathology may in fact contain micrometastases, missed due to node sectioning or human error.

Discussion

Histopathological examination, sometimes paired with immunonohistochemistry, is a basic technique used to assess lymph nodes in thyroid cancer patients, yet it does not evaluate the lymph node status precisely. Occult lymph node metastases can appear in up to 20% of thyroid cancer patients [35]. This is possibly caused by insufficient lymphadenectomy or/and micrometastases that are not found in routine histopathology. This examination has limited value because only several slides from each biological sample are checked. Serious histopathological examination is more sensitive but requires much more work to evaluate many slides, so it cannot be used as a standard procedure. Perhaps molecular methods allow the examination of all the material from lymph nodes. Kary Mullis invented the polymerase chain reaction (PCR) — a new technique which allows millions of DNA copies with required sequences to be obtained [36]. Nowadays, there are many types of PCR reactions. One of them — the reverse transcriptase-polymerase chain reaction (RT-PCR) — is often used to find metastases in lymph nodes. This method has been also tested in thyroid cancer studies [37, 38]. These studies suggest that RT-PCR is sensitive enough to detect nodal involvement in thyroid cancer. However, it is a time-consuming procedure, making it unsuitable for intraoperative examination. Real-time PCR and OSNA could be used for this purpose. Real-Time PCR is a method based on PCR, which is performed to amplify and simultaneously quantify specific DNA. The median time needed for real-time PCR testing is 40 minutes, confirming its suitability for intraoperative examination. OSNA is quite a new method that uses the reverse-transcription loop-mediated isothermal amplification (RT-LAMP) technique for gene amplification. This technique was invented by Notomi et al. [39]. The reaction is performed isothermally by means of six primers and is able to detect mRNA of CK19 without interference of pseudogenes. The assay can differentiate contamination of a few benign epithelial cells by using a verified cutoff value. It quantitatively measures the levels of CK19 mRNA), which is considered as a marker of cancer cells. OSNA was used for the first time in breast cancer patients by Tsujimoto et al. [33]. In this method, the supernatant of a homogenised lymph node solution is directly analysed without the mRNA purification process that is usually required in PCR. The use of an automated gene amplification machine permits a standardised and rapid procedure. A single examination takes 30-40 minutes, meaning OSNA can be used as an intraoperative examination. OSNA is clinically used for the evaluation of lymph node metastases in breast cancer as well as in colorectal cancer, gastric cancer and head and neck cancer [40–44]. It uses CK19 mRNA as a marker. In this study, we decided to compare OSNA and real-time PCR with histopathology as a gold standard to detect lymph node metastases of PTC. Two markers, TG and CK-19, were used for real-

time PCR while CK-19 was used for OSNA. It is a pity that there is no commercial TG marker kit for OSNA. In our study, 92 lymph nodes were examined. The results of OSNA and histopathology were different only in seven (7.6%) lymph nodes. Three lymph nodes were positive in OSNA while they were negative in histopathology. Curiously, one sample was also positive in both real-time PCRs and negative in histopathology. The three other samples were negative in real-time PCR. These results suggest that alternate parts of the lymph nodes, which may or may not enclose cancer cells, were taken for the pathology and OSNA, respectively. This is so because samples for molecular examination must be homogenised. They cannot be used for pathology, therefore studies which compare two modalities from different slices of the same lymph node must give some cases of discrepant results. This is a limitation of such studies, yet there is no way of avoiding it. It would be interesting to perform OSNA examination on the whole lymph node, as is done in breast cancer [45, 46]. Unfortunately, since no studies evaluating the accuracy of OSNA in thyroid cancer have been carried out, it is impossible, from an ethical and legal point of view, to perform OSNA instead of histopathology. On the contrary, there were four OSNA (–) and histopathology (+) lymph nodes. Interestingly, all but one of them was positive in both PCR examinations. This sample was also positive in one real-time PCR examination. These results suggest that OSNA negative results are truly negative, and that molecular examination was not sensitive enough to detect metastases in these lymph nodes. Based on this outcome, we concluded that OSNA is a sensitive technique in the detection of nodal metastases of the papillary thyroid cancer. In eight (8.7%) lymph nodes, metastases were detected with RT-PCR for TG mRNA, although the lymph nodes were classified histologically as metastasis-free. Perhaps this was caused also by a sampling error, or by illegitimate amplification, or pseudogenes. Bojunga et al. and Austin et al. described illegitimate amplification in PTC [47, 48]. The number of false positive results increases with an increased number of PCR cycles. Only one of these eight lymph nodes was OSNA (+). This indicates that the results of PCR are rather false positive but it also impossible to exclude a sampling mistake. On the other hand, in three (3.3%) lymph nodes, RT-PCR for TG mRNA failed to detect thyroid cancer cells despite positive histopathology. There were ten (9.2%) discordances between histopathology and real-time PCR for CK19. Five of them were positive only in the molecular examination, and five only in histopathology. In the subgroup with positive PCR for CK19, only one node was positive in OSNA. Perhaps these positive results of real-time PCR are caused by pseudogenes. At least two pseudo-genes for CK19, namely CK19a and CK19b, which have significant sequence homology with mRNA CK19 have been identified [48]. In the subgroup with positive histopathology, three nodes had positive OSNA result. Curiously, three lymph nodes from the same patient were histopathology (+), CK19 real-time PCR (–) but also TG real-time PCR (+). This result, and other differences between PCR examinations, reflect the heterogeneity of markers' expression in lymph node metastases of PTC. If we treat histopathology as a referential examination, then the results of OSNA and both PCR examinations are similar. No statistically significant differences in the diagnostic accuracy of either method with regard to the histopathological examination were noted. There were seven differences between OSNA and histopathology, 11 between TG real-time PCR and histopathology, and ten between histopathology and CK19 real-time PCR. Both OSNA and real-time can be performed in 35 minutes or less, and thus could be performed intraoperatively to detect metastases to lymph nodes. Real-time PCR can be very sensitive in finding cancer cells against a background of normal cells. It is able to detect histological occult micrometastases in many cancer types [14, 50–52].

Despite its advantages, real-time PCR also has its limitations. It requires mRNA extracting from lymph nodes and its purification, next reverse transcription and PCR. These procedures are prone to RNA contamination and degradation, resulting in possible false positive and false negative outcomes. Additionally, the presence of pseudogenes may lead to false positive results. On the contrary, OSNA is highly automated. It does not require sterile working conditions for denaturation and hybridisation of nucleic acid chains. Nevertheless, lymph node harvesting in fresh tissue is much harder compared to formalin fixed material and requires special training. The OSNA assay amplification directly starts from the lysate. Presumably OSNA will become cheaper. According to a Spanish cost-benefit analysis, the OSNA technique used in breast cancer reduces the number of admission days and the duration of surgery, and achieves a saving of about 440 euros per patient [53].

Conclusions

In this study, we found that OSNA is a reliable and easy technique for the intraoperative evaluation of lymph nodes in PTC. OSNA was shown to have equivalent accuracy to histopathology and real-time PCR. We anticipate that the OSNA method will be a new alternative to lymph nodes evaluation in PTC, allowing for their whole examination with a high degree of sensitivity and specificity. Based on our real-time PCR outcomes, we suppose there is a need to assess markers other than CK19 for OSNA.

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