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Content of RNA originating from thyroid in washouts from fine-needle and core-needle aspiration biopsy — preliminary study

Zawartość RNA pochodzącego z tarczycy w popłuczynach z biopsji aspiracyjnej cienkoigłowej i biopsji mandrynowej — badanie wstępne

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Abstract

Introduction: In the evaluation of molecular markers in washouts from fine-needle aspiration biopsy (FNAB) the extremely small amount of material can be a major problem. Some authors tried to use washouts from core-needle aspiration biopsy (CNABs) to gain more material from larger needles. However, according to some studies, CNAB samples are commonly contaminated with blood. The aim of our study was to evaluate the proportion of nucleic acids from thyroid cells in washouts from FNAB and CNAB by measuring the relative expression of cytokeratin 17 (KRT17) on the mRNA level.

Material and methods: Relative expression of KRT17 and GADPH (reference gene) in washouts from FNAB and CNAB was measured using real-time PCR technique and compared to the results from surgical specimens.

Results: Surgical specimens form 22 nodules, FNAB samples from 20 lesions and CNAB samples from 24 lesions were analysed. The median difference in cycle threshold (Ct) between FNAB samples and surgical specimens was 3.3 ($p = 0.047$). In CNAB samples KRT17 was undetectable in most cases (median incalculable; proportion of samples with undetectable KRT17 significantly higher than in FNAB samples).

Conclusions: Samples obtained with different biopsy techniques had different proportions of contents. The proportionally low content of epithelial cells in CNAB can result in underestimated expression of molecular markers of malignancy. Consequently, the risk of malignancy or unfavourable prognosis can also be underestimated. To conclude, results obtained from samples gained with one biopsy technique cannot be directly related to thresholds, and generally with experiences gained with other techniques, because it can lead to incorrect clinical interpretation of the results. **(Endokrynol Pol 2016; 67 (6): 550–553)**

Key words: *fine-needle aspiration biopsy; core-needle aspiration biopsy; thyroid; nodular goitre; cytokeratin 17*

Streszczenie

Wstęp: W przypadku oceny markerów molekularnych w popłuczynach z biopsji aspiracyjnej cienkoigłowej (FNAB), skrajnie mała ilość materiału może stanowić istotny problem. Niektórzy autorzy usiłowali użyć popłuczyn z biopsji mandrynowej (CNAB), aby uzyskać większą ilość materiału z większych igieł. Jednakże, według części badań, materiał uzyskany z CNAB jest zwykle zanieczyszczony domieszką krwi. Celem naszej pracy była ocena proporcji kwasów nukleinowych pochodzących z komórek tarczycy w popłuczynach z FNAB i CNAB poprzez pomiar ekspresji cytokeratyny 17 (KRT17) na poziomie mRNA.

Materiał i metody: Względną ekspresję KRT17 i GADPH (gen referencyjny) w popłuczynach z FNAB i CNAB zmierzono, używając techniki PCR w czasie rzeczywistym i porównano z wynikami z wycinków operacyjnych.

Wyniki: Przeanalizowano wycinki operacyjne z 22 guzków, popłuczyny po FNAB z 20 guzków i CNAB z 24 guzków. Mediana różnicy w cyklu granicznym (Ct) między FNAB i wycinkami operacyjnymi wyniosła 3,3 ($p = 0,047$). W próbkach z CNAB KRT17 była w większości przypadków niewykrywalna (mediana niepoliczalna, odsetek próbek z niewykrywalną KRT17 istotnie statystycznie wyższy niż w przypadku próbek z FNAB).

Wnioski: Próbki uzyskane z użyciem różnych technik biopsyjnych posiadały różną proporcję składników. Proporcjonalnie niska zawartość komórek nabłonkowych w próbkach z CNAB może skutkować niedoszacowaniem poziomu ekspresji molekularnych markerów złośliwości. W konsekwencji ryzyko złośliwości czy niekorzystnej prognozy może być również niedoszacowane. Reasumując, wyniki uzyskane na bazie próbek uzyskanych przy użyciu jednej techniki biopsyjnej nie mogą być wprost przenoszone na progi czy ogólniej, doświadczenia uzyskane przy użyciu innych technik, gdyż może to prowadzić do niewłaściwej klinicznej interpretacji wyników. **(Endokrynol Pol 2016; 67 (6): 550–553)**

Słowa kluczowe: *biopsja aspiracyjna cienkoigłowa; biopsja mandrynowa; tarczyca; wole guzkowe; cytokeratyna 17*

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Introduction

The occurrence of thyroid nodular goitre is estimated at up to 70% in the general population [1], and according to numerous studies it may be even higher in some specific sub-populations of patients at certain ages or in those suffering from some chronic medical conditions. [1–5].

Ultrasonography (US) and fine-needle biopsy remains the golden standard in the diagnostics and selection of lesions presenting high risk of malignancy, which should be surgically removed [6]. The major limitation of the method is its high percentage of non-diagnostic results, estimated to be about 10 to 20% [7–9]. Such patients constitute an important clinical problem because there is a risk of delayed treatment of thyroid cancer (TC) as well as unnecessary thyroidectomy in cases of patients with benign nodules. This problem indicates the need for additional diagnostic tools that could allow for distinction between benign and malignant lesions in cases of patients with non-diagnostic results of FNAB, especially when the result is repeated. There have been numerous studies about the use of different diagnostic tools, such as elastography, PET/CT, core-needle aspiration biopsies (CNABs), and finally evaluation of different molecular markers of malignancy, which seem to be promising [2, 10–16].

In the evaluation of molecular markers in washouts from FNAB extremely small amounts of material can be a major problem. Some authors have tried to use washouts from CNABs to gain more material from larger needles [17]. However, according to previously published clinical studies, CNAB samples are commonly haemorrhagic or contain other contaminations, such as high amounts of liquid in case of partially cystic lesions. On that basis it could be hypothesised that even if the amount of material in CNAB samples is usually visibly higher, the proportion of thyroid cells in the samples is significantly lower [10].

The aim of our study was to evaluate the proportion of nucleic acids from thyroid cells in washouts from FNAB and CNAB by measuring the relative expression of cytokeratin 17 (KRT17) on the mRNA level; KRT17 is expressed in a few types of epithelial tissues including thyroid follicular cells, but not in leukocytes, which are the most important source of extrathyroidal nucleic acids in FNAB and CNAB samples [18].

Material and methods

The Poznan University of Medical Sciences Ethical Committee approved the study. All participants were educated about the study and were asked to sign a written consent form.

Tissue samples

FNAB and CNAB

Patients were recruited from a local outpatient clinic. US examinations and biopsies were performed by four experienced sonographers (MR, AS, ESP, EG). CNABs were performed using a 22 G × 1.5'' (0.7 mm × 40 mm) core needle, FNABs — using 25 G × 1.5'' (0.5 × 40 mm) needles. After biopsy the needle was flushed with RNA Later solution (Life Technologies). The washouts were frozen at –80ºC. Material from patients with diagnostic and benign results of biopsies were selected for further analysis.

Surgical specimens

Tissue samples were obtained from patients who underwent thyroidectomy at the Department of General, Gastroenterological, and Endocrine Surgery, Poznan University of Medical Sciences in Poznan, Poland. Specimens were stored in RNA Later solution and frozen at –80ºC. Specimens from nodules with benign histopathology were selected for further analysis.

Outcomes of cytopathology and histopathology

To gain comparable material, samples from lesions diagnosed as colloid nodules were used in further analyses.

RNA isolation

RNA was isolated with AllPrep Micro Kit (Quiagen) according to the manufacturer's protocol. The amount of RNA was measured using a Qubit 2.0 Fluorometer.

Reverse transcription

Reverse transcription was performed using a RETROscript Reverse Transcription Kit (Life Technologies) according to the manufacturer's protocol.

Quantitative PCR

The StepOne Plus Real-Time PCR System (Life Technologies) was used. Expression of Cyt-17 and GAPDH (reference gene) were measured. Experiments were performed using TaqMan® Gene Expression Assays. All experiments were performed in triplicate. Samples with GADPH cycle threshold (Ct) up to 35 were consider adequate for analysis. Every real-time PCR experiment included 50 amplification cycles. ΔCt between KRT17 and GADPH over 15 was interpreted as undetectable KRT17.

Statistical analysis

Calculations were performed using Statistica 10 from StatSoft. A P level of less than 0.05 was considered statistically significant. Results were given as medians, and first and third quartile because undetectable levels of

KRT17 would influence the reliability of calculations of the mean values. In cases were medians were calculable the significance of difference was calculated using the Mann-Whitney test. In cases were medians were incalculable (KRT17 was undetectable in most cases) the proportion of samples with undetectable KRT17 levels was calculated using the χ^2-square test.

Results

Surgical specimens from 22 nodules, FNAB samples from 20 lesions, and CNAB samples from 24 lesions were analysed. In all surgical specimens KRT17 was detectable, median ΔCt was 10.5, first quartile (Q1) — 8.6, and third quartile (Q3) — 13.2. In FNAB samples KRT17 was detectable in 11 samples, median — 13.8, Q1 — 9.5, and Q3 — 15.0. The difference between surgical specimens was significant ($p = 0.047$). In CNAB samples KRT17 was undetectable in 20 samples — it was a significantly higher proportion than in the case of surgical specimens ($p < 0.001$) and FNAB samples ($p = 0.04$).

Discussion

Cytokeratin 17 is an extracellular matrix protein and a member of the cytokeratin subfamily. The KRT17gene is expressed in the basal cells of complex epithelia, e.g. in the respiratory and urinary tract and some glands, including thyroid, but not in normal epidermis [19–21]. Nikiforov et al. described the use of KRT17 in order to assess the proportion of thyroid cells in FNAB samples and compared expression of the gene in different types of thyroid nodules and in white blood cells (WBC); according to that study, KRT17 was expressed at similar levels in surgical specimens from normal thyroid tissue, and follicular and papillary thyroid carcinomas. Expression was several thousand times lower in WBC [18]. However, the issue of the expression of KRT17 in malignant thyroid neoplasms can be considered controversial. Kim et al. described significant overexpression of cytokeratin 17 in specimens from papillary thyroid cancer at protein level, using immunohistochemical stainings [19]. Overexpression of the gene was also described in several different epithelial neoplasms, such as epithelial ovarian cancer or squamous cell carcinoma of the cervix [22, 23]. We decided to include surgical specimens as well as CNAB and FNAB samples from benign thyroid lesions only.

According to our results, the relative expression of KRT17 in FNAB washouts was significantly lower in comparison with surgical specimens. The difference between the ΔCt medians was 3.3 cycles, which corresponds to over nine-times lower content of epithelial cells. This result is worth noticing: the vast majority of RNA in FNAB samples comes from extrathyroidal cells. In the mentioned study performed by Nikiforov et al. the exact data about the relative expression of KRT17 in FNAB samples was not given. However, the threshold of 3.5 cycles of difference in comparison to averaged results from normal thyroid tissue was used as a cut-off point for sample adequacy. That threshold corresponds to approximately 10% of epithelial cells. It can be assumed that even if the relative expression of KRT17 and GAPDH in normal thyroid tissue differed strongly between studies, the results concerning the proportion of epithelial cells in FNAB had the same order of magnitude.

In the case of CNABs the relative expression of KRT17 was incomparably lower. In most cases KRT17 was undetectable, which suggests a much higher proportion of RNA coming from non-epithelial tissues, e.g. from white blood cells. The exact proportion of thyroid cells could not be calculated; probably a study analysing whole CNAB samples — not only washouts — is necessary to gain more complete and precise results. However, this finding is in compliance with our clinical observations. As we reported in a previous study performed in the same department and using the same equipment, CNABs did not bring a higher proportion of diagnostic results than FNABs. However, the cytopathological descriptions of non-diagnostic FNABs and CNABs were different — in the case of FNAB it was usually a small amount of thyroid cells, whereas in the case of CNAB there was a high content of blood, colloid liquid, etc. Even samples giving diagnostic results were usually more or less visibly haemorrhagic [10]. This difference in estimated content of epithelial cells in CNAB samples can be translated into lower levels of expression of molecular markers of malignancy, which are expressed in thyroid cells, eventually into a smaller proportion of cells with somatic mutations typical for TCs. Consequently, the risk of malignancy or unfavourable prognosis can be underestimated.

To our knowledge, the current study is the first research comparing the proportion of epithelial cells in washouts from FNAB and CNAB. It is worth remembering that there is a great variety of available equipment using during CNABs — from quite simple needles which differ from conventional fine-needles according to their larger diameter and the presence of a removable inner stylet [10] to more sophisticated, but also invasive, automatic biopsy guns [24, 25]. The results of the studies comparing the risk of gaining non-diagnostic results of CNAB and FNAB are extremely heterogenous — according to some authors CNAB brought over ten-times less risk of a non-diagnostic result than FNAB [24–27], whereas other studies did not report any significant

difference in outcomes [10]. This heterogeneity could result from differences in equipment, as well as from many factors that are difficult to uniform and calculate, such as the experience of the sonographer, the number of passes during the biopsy, etc. In the case of molecular studies, another reason why results can vary is the issue of whether material for molecular analyses comes from a distinct puncture or if just washouts from needles are available.

To conclude, our results cannot be generalised too widely and be interpreted as evidence that CNAB samples are less adequate for the evaluation of expression of any molecular markers of malignancy. It should rather be concluded that samples obtained with different biopsy techniques possibly have different proportions of contents. Consequently, results obtain from samples gained with one biopsy technique cannot be directly related to thresholds, and generally with experiences gained with other techniques, because it can lead to incorrect clinical interpretation of the results.

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