IMPORTANCE OF HORMONES AND PROTEINS DETERMINATION IN THE MATERIAL OBTAINED BY FINE-NEEDLE ASPIRATION

ZNAČAJ ODREĐIVANJA HORMONA I PROTEINA U MATERIJALU DOBIJENOM ASPIRACIJSKOM PUNKCIJOM TANKOM IGLOM

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Summary: More than a half century of experience with aspiration punch of nodal changes in the thyroid gland has confirmed this procedure as a golden standard in the examination of thyroid nodal disease. Although sensitivity, specificity, reliability and reproducibility are incontestably high, this procedure cannot give a simple answer on whether the change examined is benign or malignant. Numerous attempts to improve the procedure resulted in considerably advanced findings. Besides refining the cytopathologic examination techniques, confirmation or determination of hormones, proteins and other substances in the material obtained by fine-needle aspiration are actually the greatest contribution to improvement the of procedure's diagnostic value. These markers are actually followed, in most medical centers, in aspirates of thyroid nodal changes but also surrounding lymph nodes in order to evaluate with greater certainty the type, volume and spread; this is important to establish treatment procedures and to evaluate the residual disease after accomplishing the treatment.

Keywords: fine-needle aspiration, thyroglobulin, BRAF mutation, differentiated thyroid carcinoma, thyroid nodal disease

Kratak sadržaj: Više od pola veka iskustva sa aspiracijskom punkcijom nodoznih promena u štitastoj žlezdi utvrdilo je ovaj postupak kao zlatni standard u ispitivanju tiroidne nodozne bolesti. Iako su osetljivost, specifičnost, pouzdanost i reproducibilnost dokazano visoke, ovaj postupak ipak u skoro petini slučajeva ne može jednoznačno da odgovori da li je ispitivana promena benigne ili maligne prirode. Mnogobrojni pokušaji da se postupak popravi doveli su do značajnog poboljšanja vrednosti njime dobijenih nalaza. Pored rafiniranja tehnika citopatoloških pretraga, dokazivanje ili određivanje hormona, proteina i drugih supstanci u materijalu dobijenom aspiracijom tankom iglom danas predstavlja najveći doprinos u poboljšanju dijagnostičke vrednosti postupka. Ovi belezi se danas u najvećem broju centara prate u aspiratima nodoznih promena u štitastoj žlezdi ali i okolnih limfnih nodusa kako bi se sa većom sigurnošću ocenile vrsta promene, obim i stepen proširenosti, što je od značaja u pripremi terapijskih postupaka ali i za ocenu rezidualne bolesti posle primenjenog lečenja.

Ključne reči: aspiracija tankom iglom, tireoglobulin, BRAF mutacija, diferentovani karcinom tireoidee, tiroidna nodozna bolest

Hormones and proteins in FNA fluid

Nodular changes of the thyroid structure are a frequent occurrence; they are found by palpation in around 4–7% of adults. Thyroid nodules are more frequent in female and aged individuals. Incidence of newly discovered nodules increases especially after proceeding to neck and thyroid ultrasonography; thyroid nodules are found by ultrasonography in over one third of the overall population, while some series show a presence of nodular change in the thyroid gland in over 50% of adults (1). Such a large number of nodular changes is a great economic burden on public health and an important psychological burden

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Abbreviations: FNA – fine-needle aspiration; PTC – papillary thyroid cancer; FTC – follicular thyroid cancer; MTC – medullary thyroid cancer; ATC – anaplastic thyroid cancer; Tg – thyroglobulin; PTH – parathyroid hormone ; MAP kinase – mitogen-activated protein kinase; BRAF – B raf gene for B type Raf kinase.

on patients. Since only 4–7% of all nodules are palpable and only 0.04% of all nodules found by ultrasonography are malignant, the primary objective of evaluating any nodular lesion found is to exclude its malignant nature (2). It is, therefore, safest both for patients and physicians to consider every lesion as malignant until the contrary is proven; such evidence should, of course, be accessible and reliable (3).

Epithelial Neoplasms of the Thyroid Gland

Thyroid cancer is the most frequent endocrine malignant neoplasm. Like all malignant neoplasms, it can be of epithelial and nonepithelial origin. Malignant neoplasm of thyroid epithelial cells is a thyroid carcinoma, which is usually histologically classified as differentiated carcinoma of thyroid follicular epithelial cells (DTC), carcinoma of parafollicular C cells and undifferentiated carcinoma of epithelial cells, i.e. anaplastic carcinoma. Cells of differentiated thyroid carcinoma maintain some properties of normal follicular cells, mainly the capability to take on iodine to some extent, synthesize thyroglobulin and react on the action of thyrotropin which is a natural stimulator of thyroid function and growth (TSH). Differentiated thyroid carcinomas are classified into papillary thyroid carcinoma (PTC) with several histological subtypes, and follicular thyroid carcinoma (FTC) also with several histological subtypes. A subtype of differentiated thyroid carcinoma, Hürthle cell carcinoma, is considered to be a separate variant of follicular thyroid carcinoma. The following frequencies of all epithelial thyroid malignant lesions are estimated: PTC and its subtypes – around 80%; FTC – around 15%; parafollicular C-cell carcinoma known as medullary thyroid carcinoma (MTC) – around 3%; anaplastic thyroid carcinoma (ATC) – around 2%.

Fine-Needle Aspiration of Thyroid Nodular Changes

Fine-needle aspiration of the thyroid gland and cytologic analysis of aspirate are actually the most widespread procedures of initial diagnosis of the nature of thyroid structural lesions (4). In spite of lengthy application, great experience and refined sampling and processing techniques available today, this procedure cannot always differ with certainty those malignant from numerous benign changes within thyroid structure. Around 30% of aspirate analyses do not give incontestable proof of lesion's nature and their results are classified as indefinite. Method's inherent defect is exactly the impossibility to differ benign from malignant follicular lesions since essential data for capsule differentiation and relation to blood vessel, possible only in histologic preparation, are missing. In order to improve the validity of cytologic examination and increase the reliability of results,

numerous new coloring techniques and also molecular diagnostic procedures were introduced. Since PTC is the most frequent thyroid carcinoma, although with relatively benevolent course, great efforts are made to improve diagnostics of this neoplasm and to indicate indirectly (since cytologic differentiation of cellular subvariants is impossible), and preoperatively, its possible more aggressive form enabling a surgeon to be more radical, taking always into consideration the desirable optimal relation between surgery extent and reduction of unwanted but existing treatment complication dangers. Since the administration also of iodine ablative doses of radioactive in postoperative treatment of differentiated thyroid carcinoma was actually adopted, preoperative identification of a potentially more aggressive DTC form influences a more liberal approach to the application of therapeutic radionuclides.

BRAF V600E Mutation and Thyroid Cancer

Described for the first time eight years ago (5), the BRAF V600E mutation has been proved to be the most frequent genetic alteration in sporadic PTCs (6). Diagnostics of thyroid carcinoma were considerably improved by finding this mutation in samples obtained by fine-needle biopsy. During this period around forty BRAF gene mutations were found and described; among these, the T1799 transversional point mutation in the 15th exon is the most frequent and covers over 90% of all mutations found in BRAF gene (7, 8). This mutation is frequently found in thyroid cancer (9) and provokes the V600E replacement of amino acid within the BRAF protein, which then provokes a constitutive and oncogenous activation of mutated BRAF kinase (10). A small number of other activated BRAF mutants – such as BRAF K601E (11), AKAP9-BRAF, BRAF V599ins, BRAF V600E_K601del (12), and recently described BRAF mutant V600D_FGLAT601-605ins occurring by insertion of 18 nucleotides into the BRAF gene T1799 nucleotide (13) – is rarely found in thyroid cancer. The T1799A mutation is therefore the only BRAF mutation identified in thyroid cancer. BRAF mutation represents a somatic genetic alteration; no germinal mutation was found in familial thyroid cancers (14). A very important characteristic of BRAF mutation in thyroid cancer is its exclusive occurrence in PTC and ATC originating from previously existing PTC. Estimated average prevalence of BRAF mutation is 44% in PTC and around 24% in ATC. This mutation was not found in FTC or other types of thyroid tumors or benign lesions (14). High frequency and specificity of BRAF mutation in PTC confirm its exceptional and fundamental pathogenic role in the occurrence of PTC. Search for potential clinical uses of this mutation as a new prognostic molecular marker and efficient PTC treatment goal was started with enormous enthusiasm.

Several studies evaluating the diagnostic applicability of discovering BRAF mutations in samples ob-

tained by fine-needle aspiration of the thyroid gland were carried out (15, 16). Most of these studies were retrospective, made on samples taken from microscopic plates, so that BRAF mutations were evaluated when the operated tumor's histopathological diagnosis already existed. The Xing et al. study (16) was prospective: it analyzed BRAF mutations in the material obtained preoperatively by fine-needle punch, and then the results were compared with postoperative histological nodular findings. Disregarding the BRAF mutation discovering procedures applied, all these studies show an exceptional reliability and simplicity of discovering a BRAF mutation in samples obtained by fine-needle thyroid aspiration. Sensitivity and specificity of BRAF positive PTC samples obtained by FNA were 100%. Conformably to findings on FNA-obtained samples, BRAF mutations were recorded only in histologically confirmed PTCs and were not found in FTCs or benign tumors. These studies indicated a 44% prevalence of BRAF mutations in PTCs, similarly to the general prevalence of this mutation. It is therefore supposed, and confirmed by these FNA studies, that almost half of these PTC patients can be diagnosed only on the basis of BRAF mutation analysis in fine-needle biopsy samples. If the BRAF mutation's diagnostic value is confirmed in more studies other than these, a PTC diagnosis based only on finding a BRAF mutation will probably not require further diagnostic cytologic studies. Finding a BRAF mutation is reliable and not costly (15), especially if effected by adequate procedure in centrally coordinated laboratories. Taking into consideration the important prevalence both of BRAF mutations and papillary carcinoma, elimination of a need for cytology in almost half of PTC patients could largely diminish costs of treatment. Finding a BRAF mutation also enables a more specific diagnosis of PTC since considerable intraobserver and interobserver variations exist in cytology (17). Baloch et al. indicate in their study (15) that 13% (7 of 53) FNA samples were cytologically marked as benign, and that 7% (1 of 14) FNA samples diagnosed as thyroiditis were BRAF positive, so the diagnosis based on mutation analysis was corrected into PTC. However, only the BRAF mutation diagnostic in FNA samples is not sufficient to solve the dilemma of nondiagnostic and indefinite cytologic findings. Meta-analysis of data from a number of independent studies showed that in 17% of cytologically marked nondiagnostic findings thyroid carcinoma could be discovered by finding a BRAF mutation. When all indefinite cytologic cases were evaluated as a whole, only a small number of patients, around 8%, were diagnosed by discovering a BRAF mutation, because most thyroid tumors of indefinite cytology are benign – follicular adenoma or, much more rarely, BRAF negative carcinoma; only around 15% of cytologically indefinite thyroid nodules were PTC confirmed (18). Taking into consideration the general prevalence of BRAF mutation of around 45%, 15% PTCs in cytologically indefinite findings could be translated into around 7% positive BRAF mutation findings (as found by analyzing recently some indefinite CP results).

Positive BRAF mutation is of exceptional predictive value and can be a PTC diagnosis; on the other hand, negative findings on patients have no diagnostic value at all. It is therefore necessary to finally evaluate the efficiency of BRAF mutation analysis in thyroid nodule aspirates to solve the diagnostic dilemma of indefinite cytologic findings. Around 300,000 new thyroid nodules per annum are discovered in the United States (19). If all these nodules were evaluated by biopsy, 30% of indefinite findings would mean around 90,000 indefinite nodules. Diagnostic sensitivity of 8% in discovering a BRAF mutation in cytologically indefinite findings means that around 7,200 patients could benefit from a final PTC diagnosis and proceed to optimal treatment. There are no data for our country, but it is evident that, on a considerably smaller number of samples, only a comprehensive centralized research within this country as a whole would be scientifically and economically justified.

Testing of BRAF mutation in cytologic samples from plates, when conservative follow-up of clinically indefinite cytologic findings is uncertain, could be useful and commercially justified. Combining BRAF mutation with other sensitive and specific molecular markers is probably the next approach which will increase the diagnostic sensitivity of FNA. Such an approach was recently verified by a combined use of BRAF mutation and RET/PTC analyses (20) which showed to be a procedure that really improves diagnostic sensitivity. Such verifications should be repeated on larger series since RET/PTC is sometimes found also in benign tumors (21, 22). The combination of BRAF mutation and RAS mutation analyses together with fine-needle biopsy results analysis (15) has the same limitations as the analysis of RAS mutations (23). Since cancer cells can also pass into blood, efforts were made to find sensitive methods which could identify a BRAF mutation in DNA samples from serum. Single-chain DNA polymorphism conformation technique was used recently to discover a BRAF mutation in plasma DNA, but it seems that required sensitivity was not reached (24). Allele specific amplification was also tested in real time, enabling discovery of mutated alleles in a DNA sample, but such sensitivity is probably insufficient to discover mutated BRAF alleles in a blood sample. Lilleberg et al. (25) described recently the use of PCR amplification specific for a mutant allele which is followed by detection with HPLC platform denaturation, using post separative fluorescence to detect mutated alleles which represent under 0.1% of total analyzed DNA. This procedure enabled BRAF mutation and various RAS mutations to be scanned in plasma DNA of patients with colorectal carcinoma; 100% sensitivity was achieved. This procedure has not been applied until now on individuals with thyroid cancer. Gap-LCR

(ligase chain reaction) technique proved to be a more sensitive procedure capable to discover point mutations in the presence of over ten thousand times larger quantity of natural DNA (26). There are still no data if this procedure was applied also on samples of thyroid cancer patients. Rosenberg et al. (27) discovered by means of an even more sensitive technique of specific PCR sequences in real time a heterozygote BRAF mutation positive cell within a mixture of 21,692 normal cells. When authors applied this technique on blood samples, they proved a circulating BRAF mutation in one of five tested PTC patients. Hope that a sensitive and specific method of discovering a BRAF mutation in blood will considerably simplify diagnostics in a large number of patients with thyroid nodules is justified; positive BRAF mutation in blood will considerably reduce the need for other diagnostic tests and procedures and orientate patients directly towards suitable therapies.

Galectin-3

Tens of molecules participating in carcinogenesis, at least fifty until now, were proposed as thyroid malignancy markers (oncogenic products, altered enzymes, integrins, cadherins, lectins, etc.) (28). Among these, telomerases (29), HMGI(Y) (30), HBME-1 (31), immunoaltered thyroid peroxidase (TPO) (32) not recognized by specific antibody MoAb47 and galectin-3 are markers in which greatest hopes are put to help preoperative differentiation of benign from malignant thyroid lesions (33) in fine-needle aspirates. Galectin-3 polypeptide is a member of the family of proteins attached selectively to oligo-saccharides, known as lectins (34). Galectin-3 lectins have an important role in cell-cell and cell-matrix interactions (35), extracellular matrix organization (36), mRNA splitting (37), cell growth and apoptosis (38), neoplastic transformation and metastasizing. These lectins are expressed in cancer thyrocytes and do not exist in normal follicular cells or follicular adenoma cells (40). Their value in forecasting a lesion's malignant nature was first shown on cytologic samples (41). After initial observations, some studies doubted about full reliability of these lectins as proofs of lesion's malignant nature especially in a younger population (42). Some of these disagreements are attributed to uncertainties originating from the RT-PCR technique applied (43), immunohistochemical biotin detection systems (44), and/or monoclonal antibodies being against non-human galectins-3 but cross-reacting with human ones. Use of biotin-based methods and type unspecific monoclonal antibodies could potentially lead to wrong interpretation because of follicular cell unspecific antigenicity interference.

Telomerase Activity

Discovery of telomerase activity (TA) is considered promising in better distinguishing benign from malignant lesions (45). Telomerase activity was proven in 85–90% of frozen tumors (46) mostly endocrine-dependent (neoplasms of thyroid, parathyroid and adrenal glands, but also breast and prostate neoplasms and neuroblastoma) (47). This activity can also be found in biologic fluids such as pleural (48) and bronchial (49) lavage, urine (50) and breast fine-needle aspirate (51). In spite of high specificity, false positive results are also found, in patients with Hashimoto or lymphocytic thyroiditis (52).

Around 1–46% of thyroid neoplasms show lymphocytic infiltration (53) but lymphocytes cytologically easily differ from follicular cells and the problem of lymphocyte effect on TA specificity is lessened to a certain extent. Besides, TA determination in the material obtained by preparation washing after Diff-Quik staining helps to better determine whether TA originates from lymphocytes or neoplastic cells (54). Determination of telomerase activity can increase FNA sensitivity when the cytologic picture is indecisive or the cytodiagnostic material is poor. Since positive TA in negative conventional cytology is rare, TA cannot be used for usual procedure with thyroid nodules, and cannot be used if cytologic results indicate a large quantity of lymphocytes.

Value of Biochemical Procedures To Discover Tumor Markers in the Lymph Node Aspirate

The prognosis of patients with DTC adequately treated surgically and with ablative doses of radioactive iodine is very favorable. However, the disease recidivates locally or regionally in around 5–20% of patients who accomplished a full treatment regime. Locoregional metastases most often spread to lymph nodes of the neck. A reliable diagnosis of lymph nodes being attacked is therefore of great importance in the follow-up of patients with DTC and especially PTC which has a very pronounced tendency to spread lymphogenously and early produce metastases into lymph nodes. When altered or suspect lymph nodes are found by palpation or ultrasonography in central or lateral areas of the neck it is obligatory to proceed to their fine-needle biopsy. After initial enthusiastic attempts of palpation-controlled biopsy, ultrasound-controlled biopsy is now compulsory. However, even with high-resolution ultrasonography 6–8% of samples are false negative. Attempts were, therefore, made to improve lymph node FNA diagnostic value and reliability; use of cytologic markers was expensive and insufficiently reliable. Then Pacini et al. (55) proposed in 1992 thyroglobulin (Tg) confirming in the fine-needle aspirate. Several studies recently reported that confirming and determining thyroglobulin in the fine-needle aspirate is of higher sensitivity and specificity than aspirate's cytologic examination (56–60). According to these reports, although a precise value of thyroglobulin detection threshold was

not established with certainty, sensitivity of the procedure in discovering lymph node metastases in DTC and PTC treated patients was 100%, while sensitivity of cytology only amounted to around 85%. Cignarelli et al. (61) found that FNA-Tg helps in making a diagnose when a material poor in cells is obtained, which frequently occurs in cystic metastases. Specificity of FNA-Tg is generally estimated at 89.4–95.8% depending on the lower limit (threshold) value, and amounts to 77.3% when only FNA is used; FNA sensitivity rises up to 92.4–96.6% when combined with Tg determination (FNA-Tg). Besides, this procedure does not require separate or repeated punch, but only some time for sample preparation; it is effected very easily. Various threshold values for determining Tg in aspirate are actually used since Tg can be also found in the aspirate of nonspecifically inflammed nonmalignant nonmetastatic lymph nodes if a thyroid gland exists. It is therefore necessary to determine a threshold value above which a test is considered DTC metastasis suspected. Pacini et al. (55) set the threshold at 21.7 ng/mL FNA-Tg, a value they obtained as +2 SD findings in negative patients. Most studies following this one used this value (62, 63).

Other studies used as threshold value the highest level of Tg obtained in the aspirate of incontestably inflammation-reactive lymph nodes (64), or used the lower limit of test's functional sensitivity (0.9–1.0 ng/mL) (65) for Tg level in serum. These values have some limitations. If aspirates taken from persons with high Tg level in circulation are blood contaminated or if the needle went through the thyroid tissue during the procedure, Tg value measured can be higher even if lymph nodes were not metastasized. In operated patients, if it was not proceeded also to ablation with radioactive iodine, Tg in serum can remain measurable or even increased and contamination may cause confusion. In some cases there is a considerable overlap of Tg values obtained in lymph node benign changes with values in aspirate from nodes with proved metastases; procedure specificity is then considerably reduced.

Importance of Parathyroid Hormone Determination in Thyroid Lobe Cystic Lesions Aspirate

Cystic lesions are frequent in neck anterior loge. Besides congenital cysts which are most frequently the consequence of colloid retention in thyroglossal channel remnants and usually show in younger age, cystic degenerations of thyroid nodes, lymph nodes and parathyroid tumors are also frequent.

Parathyroid cysts were considered rare: less than 300 cases were described after Sandstrom's initial description 130 years ago. The first large series of neck ultrasonographic examinations searching for real prevalence of parathyroid cysts in the neck took place in 2009 (Capelli et al.); in contrast to previous studies

on a small number of individuals, this one recorded that parathyroid cysts are really rare and are found in only 0.075% accidentally chosen persons. By fluid aspiration from these cysts both cytologic findings and material for parathyroid hormone determination were obtained. Values were high in all samples and amounted to 111–456 ng/mL, 221 ng/mL on average. Ultrasonographic particularities of these cysts could not differentiate them from any other cystic changes in that area, and only the parathyroid hormone determination decided the cysts' real nature (66).

Calcitonin Determination in Fine-Needle Biopsy Aspirate

When MTC is diagnosed it already produced local or even distant metastases (67). Serum calcitonin is a sensitive marker of disease of C-cells secreting calcitonin. Increased calcitonin was adopted as an MTC marker, and is used in this tumor's screening. Two large thyroid associations, European and American, somewhat differ as recommendations for calcitonin determination in search of MTC are concerned. While Europeans undoubtedly recommend routine determination of calcitonin at least for the first evaluation of thyroid nodes, Americans, after initial negation, moderate now their position and leave to clinicians to decide should they use calcitonin in thyroid node evaluation. When increased calcitonin is found in blood, some authors (68) do not suggest FNA because of its relatively low specificity. In order to improve FNA usability, specificity and reliability in differentiating calcitonin limit values in serum, Massao et al. (69) determined calcitonin in thyroid node fine-needle aspirate. Although the number of patients was relatively small (27 individuals), these authors concluded that this procedure does not increase fine-needle aspiration's diagnostic value and that its value is considerably lower than determination of PTH in cystic nodes or thyroglobulin in lymph nodes. Consequently, in spite of other studies' findings (70, 71), these authors do not recommend measuring calcitonin in thyroid aspirate as a routine method. It is obviously necessary to examine the value of this procedure in larger series and multicentric studies, since it is not possible to collect a sufficient number of samples in one center because of a relatively small prevalence of medullary carcinoma.

It can be concluded that fine-needle biopsy and cytopathological examination are actually a golden standard in diagnostics of the nature of thyroid nodal disease. The possibility to determine markers specific for some malignant thyroid tumors in obtained aspirates by using refined laboratory procedures is promising but insufficiently studied.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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