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Use of molecular and genetic testing of BRAFV600E mutation for fine-needle aspiration cytology of thyroid gland

Relevance. *The growing interest to non-invasive methods of early preoperative verification of thyroid tumours requires further refinement of the traditional morphological methods of thyroid tumour diagnostics using molecular-genetic studies. The detection of BRAF genetic mutation in the material under study is a decisive additional method at this stage. According to foreign researchers, the BRAF gene mutation is most frequent in papillary thyroid cancer. BRAF mutation was found in 38-69% samples of histological material of patients operated for thyroid cancer. It was also found in up to 83% of cases in Fine Needle Aspiration (FNA)-washouts from the syringe and puncture needles obtained from patients with papillary thyroid cancer. No literature data from Kazakhstani researchers could be found regarding BRAF gene mutation detected in FNA biopsy materials in papillary thyroid cancer.*

Purpose of the study *is to identify the significance of BRAF mutation among Kazakhstani patients with nodular goiter to exclude papillary thyroid gland cancer.*

Results. *According to PCR in the form of dispersion of amplification products, positive BRAF gene mutations were detected in patients with diagnosed or suspected papillary cancer. The graphical characteristics were reflected on tracks H01, A03, B03, and C03. Track F03 shows the internal control for BRAF gene positive mutation. Other relevant columns were negative for BRAF gene mutations.*

Conclusions. *The detection of BRAF gene mutation in washouts from the puncture needle obtained from patients with thyroid nodules can be used in preoperative diagnostics for prognosis and treatment of papillary thyroid cancer.*

Keywords: *thyroid nodules, Fine Needle Aspiration (FNA) biopsy cytology, papillary thyroid cancer, BRAF marker gene.*

Introduction. Cytopathological fine-needle aspiration biopsy (FNAB) performed according to Bethesda classification is used to diagnose malignant and benign thyroid gland formations at the pre-surgery stage and contributes to systematization of the choice of further tactics of diagnostics and treatment. Firstly, the survival and mortality rates are directly dependent on a reliable and properly performed pre-surgery differential diagnostics of thyroid cancer (TC). To date, pre-surgery diagnosis is precise only in 54-61% cases [1]. Secondly, the frequency of both malignant and benign thyroid gland formations is growing every year all over the world. Thus, the TC incidence has grown by 310% from 1950 to 2004 [2]. At the same time, benign nodular thyroid gland formations (NTGF), such as colloid and hyperplastic nodes, cysts, nodal foci of lymphoplasmacytic infiltration are found in 80% of the population [3]. Thirdly, the relevance of proper differential diagnostics is proven by the fact that about 30% of NTGFs are malignant, and TC in 90% of cases develops as a nodular goiter, especially at the initial stages [4]. It should also be noted that the currently used methods of NTGF differential diagnostics at the pre-surgery stage often do not allow reliable diagnosing and providing the necessary volume of surgical treatment [5].

The currently used traditional methods of diagnosing malignant and benign thyroid gland formations shall be improved. Ultrasound, sonoelastrography, biochemical and hormonal analyzes used at the initial stage provide evidence only on the presence of nodule formations. In the past few years, fine-needle aspiration puncture biopsy (FNAB) has been the best method for distinguishing between malignant and benign nodes at the pre-surgery stage [6]. FNAB is performed under strict ultrasound control. FNAB is highly safe and low invasive, ensures high diagnostic accuracy and is not expensive. Still, FNAB does not provide enough evidence for choosing a treatment tactics for follicular neoplasia and undetermined neoplasms [7]. At the last stage, especially in undetermined situations, molecular genetic studies are used. At this stage, the presence of TC is confirmed by immunohistochemical analyzes (IHC). However, IHC is not always sufficient to determine a particular type of TC. It can be supplemented by BRAF genetic mutation determination in the test material. Some foreign researchers note the highest frequency of BRAF gene mutation in papillary TC [8]. In the study of histological material, 38–69% of patients operated for TC had BRAF mutations [9]. In the study of FNAB swabs from the syringe and puncture needles, up to 83% patients with papillary TC had BRAF gene mutation [10]. The literature search on BRAF gene mutation in papillary TC in Kazakhstan returned no result.

The use of high-tech methods of molecular genetic cancer diagnostics has significantly grown over the past decade. Molecular analyzes of biological tumor samples allow studying a whole variety of DNA markers even in the minimal content. One of such promising methods of genetic diagnostics based on the analysis of data obtained in the study of genetic markers of tumor is polymerase chain reaction (PCR). Several generations of PCR technology are known till today, the most advanced is the third generation. These methods, like Droplet Digital PCR, allow an accurate quantitative DNA assessment.

Purpose of this study is to identify the significance of BRAF gene mutation in patients with nodular thyroid gland formations to exclude papillary TC in Kazakhstan.

Materials and Methods. The article provides the results of research of 122 patients who independently applied to the Department of Endocrinology of the joint university clinic of S.D. Asfendiyarov Kazakh National Medical University in Almaty for nodular goiter. Cytological studies were performed at KazIOR. Average age of patients – 49.5 years; men to women ratio – 1.2:10. All the patients underwent aspiration biopsy of NTGF under ultrasound control.

In the course of our study, we collected samples with cytological opinions corresponding to the Bethesda categories (BSRTS 2010). Grouping of patients: Group 1 – papillary TC of category 6 (malignant tumor of thyroid gland). Group 2 – non-definitive neoplasm of category 5 (suspected cancer). Group 3 – follicular neoplasia, category 4. Group 4 - colloid goiter, category 2 (nodules of benign nature). The grouping of patients by cytology results is presented in Table 1.

Table 1 – Grouping of patients by cytology results

FNAB results	Papillary TC, Category 6	Suspected TC, Category 5	Follicular neoplasia, Category 4	Nodular colloid goiter, Category 2
Number of patients	2	1	3	116

BRAF gene mutation detection in the puncture biopsy material of all groups of patients was performed in the mo-lecular genetic laboratory of Nagasaki Medical University (Japan).

NA extraction for BRAF gene mutation detection.

Preparatory stage.

After biopsy, the contents of the puncture needle were carefully washed with 0.7 ml of RNA later solution and placed in a sterile 1.5 ml Eppendorf vial. The vial was left for half an hour at ambient temperature. Tightly closed vials were placed in a freezer for long-term storage at -20°C.

DNA isolation.

The genetic material was isolated according to the study protocol using the "QIA amp DNA Mini Kit only" (QIAGEN, Germany). 50 µl of aqueous solution containing the required DNA was obtained for isolation. After the isola-tion, DNA samples were stored at -20°C.

Determination of DNA concentration on NANO-Drop UV-View spectrophotometer.

After standard preparation of the spectrophotometer according to manufacturer's instructions, 2 µm from each sample were transferred to the special reading device to define DNA concentration.

PCR analysis.

Droplet Digital PCR was performed using QX200 Droplet Digital PCR System (Bio-Rad, USA) in four stages according to the instructions. At first stage, the samples together with the necessary primers were placed in the plastic cartridge of QX200 droplet generator. For the second stage of droplet generation, a special mineral oil was added to the relevant cartridge channels. The filled cartridge was installed into QX200 droplet generator. The samples and oil mixed in micro channels inside the cartridge to produce an emulsion – about 20,000 monodisperse droplets of 1 µl for each of the prepared samples. The third stage included the main polymerase chain reaction. All emulsion samples were transferred to a stand-ard PCR plate and amplified using a standard C1000 Touch thermocycler for a set time in standard conditions including initial denaturation and the cycles of denaturation, primer annealing, and final elongation. The final stage included the reading and analysis of obtained results. After PCR, the plate was loaded into a QX200 droplet reader to analyze the fluorescent signal. The drops were analyzed one after another to detect the target. The results were analyzed automatically by ddPCR Software.

Results and Discussion. In numerous studies on this problem, the presence of BRAF mutation was determinant for choosing the tactics of treatment of thyroid gland tumors [11, 12]. Standard morphological studies in biopsy specimens in some cases cannot give an unambiguous answer about the nature and prognosis of the tumor to allow the clinician to make a definite decision. In such cases, the presence of BRAF gene mutation definitely indicates the aggressive nature of the neoplasm. Such neoplasm requires more careful observation over time and, if necessary, prescription of a complex treatment or other more radical measures.

The results of the analysis are shown in Figure 1. Categories V-VI samples were positive for BRAF gene mutation. Category II samples had no BRAF gene mutation.

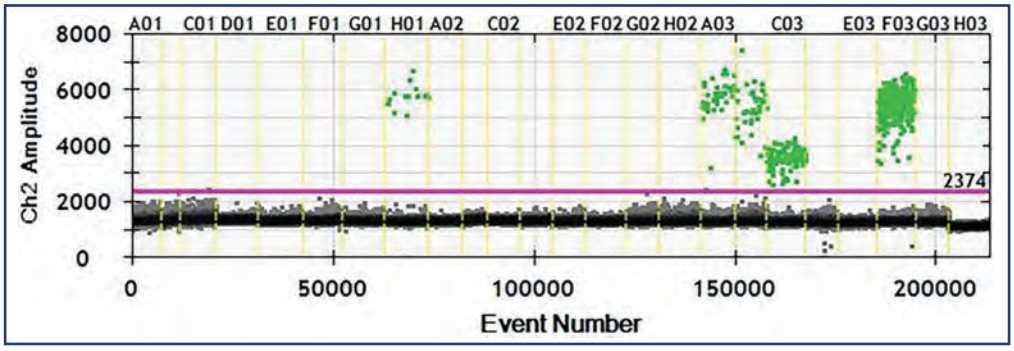

Figure 1 – Amplification products dissemination diagram. H01, A03, B03, C03 tracks – positive for BRAF gene mutation; F03 track – the internal control for positive BRAF gene mutation; other columns – negative for BRAF gene mutations.

Conclusions

1. The presence of BRAF gene mutation is a diagnostic marker for highly differentiated thyroid cancer and a high probability of malignancy of the process that will require more radical treatment approaches. The absence of this mutation is characteristic only for benign diseases that require only conservative therapy and observation over time.

2. The detection of BRAF gene mutation using the most advanced Digital Droplet PCR method can be used as an additional method for pre-surgery diagnostics of highly differentiated thyroid cancer.

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