

Two unusual epithelial cell phenotypes allow to detect papillary carcinoma in fine-needle aspirates of the thyroid

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Abstract. Fine needle aspiration (FNA) biopsy is the most commonly used method for diagnosing papillary thyroid carcinoma (PTC) and other thyroid lesions. However, cytomorphologic markers of PTC currently used in diagnostic FNA cytology are insufficiently specific. They are observed in both malignant and benign lesions, expressed by cells of not every PTC subtype and by a varying fraction of the tumor cell population. **The aim** was to examine new morphological markers of PTC applicable in FNA smears of a thyroid gland material. **Material and methods.** The study material included FNA smears of nodular goiter (n=620), follicular thyroid adenoma (n=332), Hashimoto's thyroiditis (n=54), oncocytic cell lesions (n=50), follicular thyroid carcinoma (n=22), medullary thyroid carcinoma (n=42), and PTC (n=280). FNA smears from patients of both sexes (1164 women and 236 men) aged from 17 to 87 years were examined. To analyze them cytochemistry, immunocytochemistry and computer morphometry were applied. **Results.** Two unusual epithelial cell phenotypes were identified in FNA smears of PTC but never in smears of benign and non-PTC malignant lesions of the thyroid. Cytokeratin (CK) immunocytochemistry indicated the epithelial origin of these enlarged and pleomorphic cells. Key features of the first cell phenotype included a clear cytoplasm, sharply-delineated cell borders strongly refracting light, low or negative reaction for thyroglobulin, and epCAM-negativity. Cells of the second phenotype possessed a RNA-containing macula, displayed thyroglobulin-positivity and specific pattern of epCAM expression. The question whether cells of the two identified phenotypes are related cell forms is open to discussion. **Conclusions.** The cells revealed are observed only in PTC and, thus, can be used as highly specific morphological markers having a potential to improve the reliability of PTC detection in Giemsa-stained conventional FNA smears of the thyroid. The unusual morphology of these cells permits their easy identification without time-consuming immunocytochemistry.

Keywords: diagnostic cytology, fine-needle aspiration, papillary thyroid carcinoma.

Papillary thyroid carcinoma (PTC) is the most common subtype of thyroid cancer and FNA is a first-line procedure to detect PTC along with other thyroid lesions [1-3]. In FNA cytology, the diagnostic criteria for PTC deal primarily with nuclear features and include an enlarged size, irregular shape, powdery chromatin, nuclear grooves, and intranuclear pseudoinclusions [4, 5]. Features like a papillary cell pattern, squamoid cytoplasm, psammoma bodies, and others are thought to be less diagnostically important [2, 4].

PTC diagnostic markers currently used in FNA smears are not absolute being observed both in malignant and benign lesions, expressed not in every PTC of a particular subtype and by a varying fraction of tumor cell population. Reflecting mostly the level of genomic instability, these markers are believed to be less indicative for malignancy than such histopathologic features as vascular or capsule invasions.

Generally, cancer development may be viewed as a process driven by essentially blind somatic-cell mutations [6]. The resulting traits of cancer cells reflect the impact of subsequent clonal selection [7]. Some of these traits directly relate to invasive/metastatic properties while others lack such relation. However, even features from the latter category may be diagnostically useful if they are reproducibly displayed by cancer cells and easy to identify.

The aim of this study was to search for new morphological markers of PTC applicable in FNA smears of the thyroid.

Material and methods

The study population included FNA smears collected for patients from Ukraine who were examined, diagnosed with a tumor and underwent thyroid surgery in the State Institution «V.P. Komisarenko Institute of Endocrinology and Metabolism of the NAMS of Ukraine» between 2005 and 2021. A total of 1400 FNA specimens were selected for this retrospective study. These included: nodular goiter (n=620), follicular thyroid adenoma (n=332), Hashimoto's thyroiditis (n=54), oncocytic cell lesions (n=50), follicular thyroid carcinoma (n=22), medullary thyroid carcinoma (n=42), and PTC (n=280). The patients were of both sexes (1164 women and 236 men) with their age ranging from 17 to 87 years. All reviewed cases had a histo-

pathologic diagnosis. Postoperative study of paraffin sections stained with hematoxylin and eosin was performed in the Laboratory of Morphology of the Endocrine System, State Institution «V.P. Komisarenko Institute of Endocrinology and Metabolism of the NAMS of Ukraine». The histopathologic diagnosis was made according to the WHO classification of tumors [8].

FNA of the thyroid was performed under ultrasound guidance using a 21-gauge needle, usually without a syringe or suction. Dried FNA smears were fixed for 5 minutes in methanol followed by staining in Giemsa dye diluted in 0.067M phosphate buffer (pH 6.4) for 30 minutes. Before identifying polysaccharides with periodic acid Schiff reaction, smears were destained using 5% acetic acid. To analyze the chemical composition of intracellular maculae, FNA smears were destained using 5% acetic acid, treated with RNase (Sigma, 1 mg/10 mL, 1 hour at 37 °C), and restained by Giemsa dye. Immunocytochemistry was performed in representative Giemsa-stained smears. Antigens were unmasked as described previously [9]. Endogenous peroxidase was inactivated by incubation in 1% H₂O₂ in phosphate buffer (pH 7.4) at room temperature for 30 minutes. To identify certain antigens we applied indirect immunoperoxidase technique using primary monoclonal mouse antibodies against leukocyte-common antigen (CD45) (dilution 1:50, clone PD7/26 and 2B11, Dako, Denmark), macrophage antigen (CD68) (dilution 1:50, clone EBM11, Dako, Denmark), thyroglobulin (dilution 1:100, clone DAK-Tg6, Dako, Denmark), epithelial cell adhesion molecule (epCAM) (dilution 1:100, clone Ber-Ep4, Dako, Denmark) as well as against a number of CKs, namely CK7 (dilution 1:50, clone OV-TL 12/30, Dako, Denmark), CK8 (dilution 1:100; clone M20, Sigma, USA) and CK20 (dilution 1:50, clone KS20.8, DBS, USA). Polyclonal rabbit antibodies were used to detect calcitonin (dilution 1:100, DBS, USA).

Polyclonal rabbit anti-mouse and goat anti-rabbit antibodies conjugated to horseradish peroxidase (in both cases dilution 1:100, Dako, Denmark) were applied as enzyme-labeled secondary antibodies. 3,3'-diaminobenzidine tetrahydrochloride (Sigma, USA) was used as a chromogen.

Digital images of cells were captured at resolution of 2048×1536 pixels using an Olympus CX41 microscope (Olympus Corporation, Japan) fitted with a DP12 camera (Olympus Corporation,

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Japan) and UplanFI $\times 40/0.75$ objective. Images of cells of interest were captured before and after cytochemistry or immunocytochemistry, using recorded coordinates to relocate these cells.

The area of a cell profile was selected as a measure of cell size, while the area of a nuclear profile was chosen to characterize the size of a nucleus. Measurements were made in digital images using Image J (v.1.54g, National Institutes of Health, USA). In total, 200 cells were analyzed per group. Values are shown as $M \pm SE$ of the mean. The non-parametric two-tailed Kolmogorov-Smirnov test was used to assess the differences between samples. $P < 0.01$ was considered statistically significant.

The study was performed in accordance with the standards of the bioethical committee of IEM (permit dated April 1, 2021, №35/5-KE), of the main provisions of the «Convention on the Protection of Human Rights and Dignity in the Application of Biology and Medicine: Convention on Human Rights and Biomedicine», adopted by the Council of Europe on April 4, 1997 of Good Clinical Practice (GCP) from 1996, the Declaration of Helsinki of the World Medical Association «Ethical Principles of Medical Research with Human Subjects», adopted in June 1964 and revised during 1975-2008 and the Order of the Ministry of Health of Ukraine «On the approval of the Procedure for conducting clinical trials of medicinal products and examination of clinical trial materials and the Standard Regulations on the Commission on Ethics» dated February 13, 2006 №66 with changes in 2006-2008.

Results and discussion

The study of FNA smears of PTC revealed follicular cells arranged in tissue fragments and singly, blood cells, macrophages (histiocytes) as well as more rare fibroblasts and endothelial cells. Some smears contained enlarged, mildly basophilic, presumably epithelial cells. A noticeable fraction of the latter displayed either of two distinct, partly overlapping phenotypes.

Cells of the first phenotype had a rounded shape and sharply delineated cell borders strongly refracting light (**Figure 1a**). For this reason, we named them 'refractive surface cells' (RSCs). In Giemsa-stained smears, their cytoplasm was mildly basophilic centrally and colorless at the periphery. Due to this, RSCs appeared lighter-colored with respect

to other cells. They were arranged as isolated single cells, cells integrated into monolayers of an irregular cell pattern, or isolated cell aggregates.

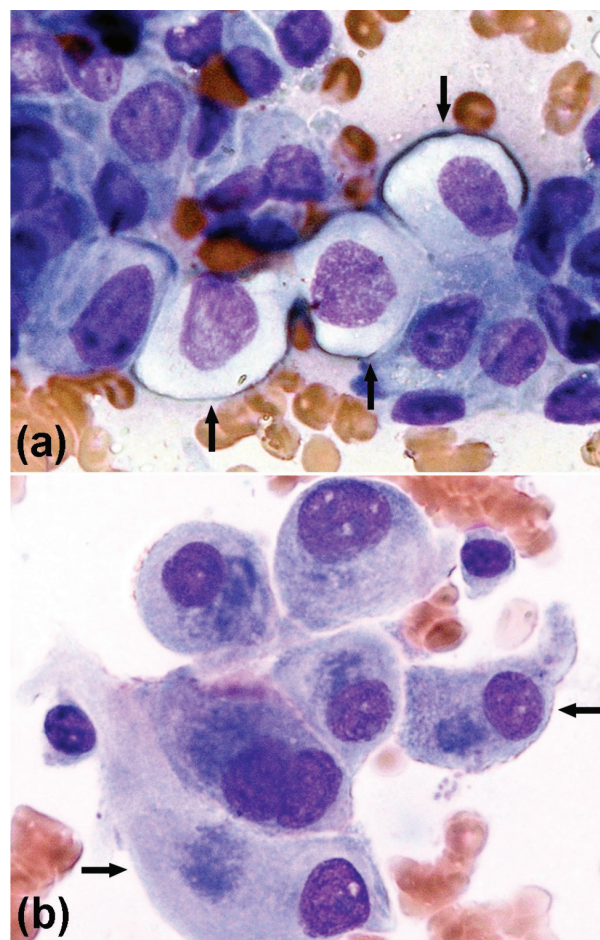


Fig. 1. 'Refractive surface cells' (arrows) (a; $\times 1000$) and 'macula cells' (arrows) (b; $\times 1000$) in FNA smears of PTC.

RSCs were more than double the size of other PTC epithelial cells, i.e., $626.2 \pm 21.7 \mu\text{m}^2$ vs $258.9 \pm 5.6 \mu\text{m}^2$ ($p < 0.01$) (**Figure 2a**). RSCs had rounded, pear-shaped or pleomorphic nuclei with a powdery chromatin and occasional intranuclear pseudoinclusions. The latter were revealed in 100% (25/25) of PTC lesions containing RSCs. RSCs' nuclear size equaled $168.2 \pm 4.1 \mu\text{m}^2$ being significantly larger than that of other PTC epithelial cells – $101.9 \pm 1.4 \mu\text{m}^2$ ($p < 0.01$). Sometimes, micronuclei were observed. The cytoplasm of RSCs had no metachromatically stained vacuoli typical of active follicular cells but sometimes contained a macula, the characteristic feature of another cell phenotype (detailed below). RSCs' cytoplasm showed no oxyphilia typical of oncocyctic cells.

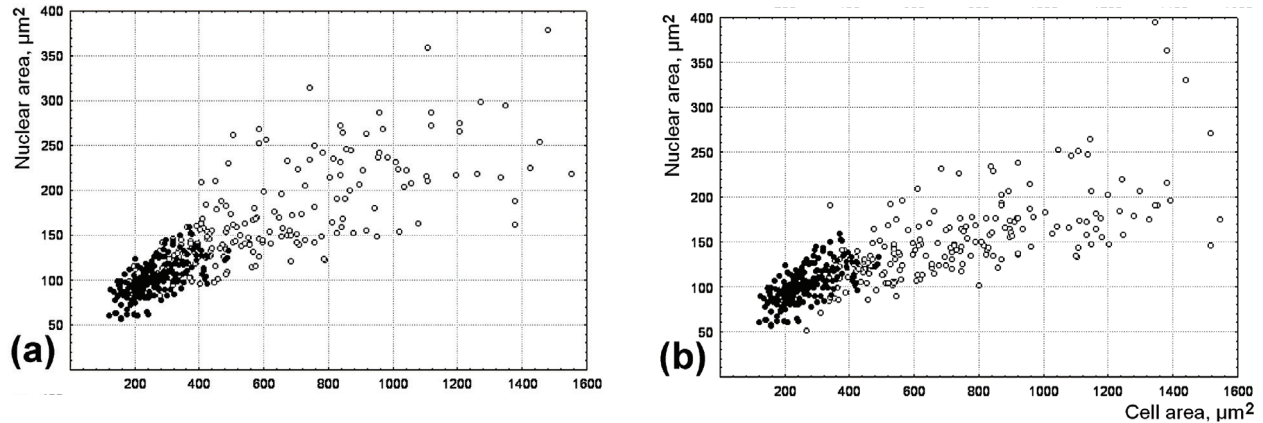


Fig. 2. Dot plots illustrating distributions of cell area and nuclear area values for 'refractive surface cells' (empty circles) (a), 'macula cells' (empty circles) (b) and other PTC epithelial cells (filled circles) (a-b).

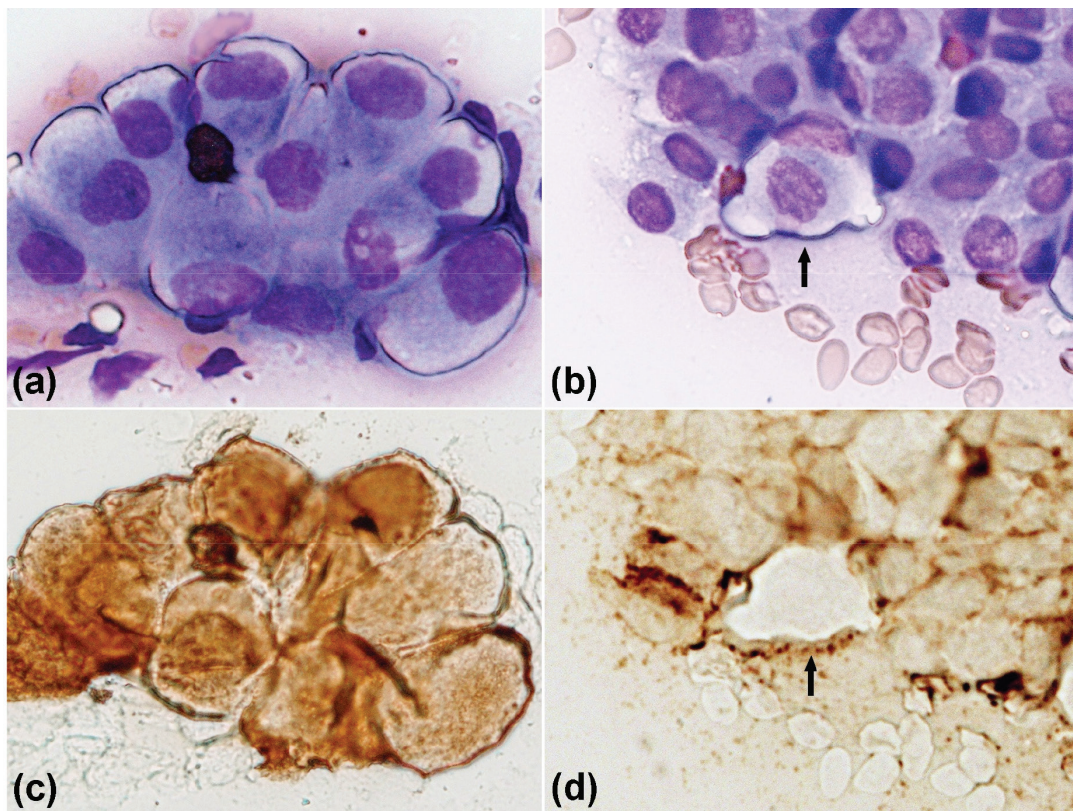


Fig. 3. 'Refractive surface cells' in FNA smears of PTC (a-d; $\times 1000$). CK7-positivity provides the evidence of the epithelial origin of these cells (c). In striking contrast to other PTC epithelial cells they are epCAM-negative (arrow) (d).

RSCs occasionally developed lobopodia-like structures and mimicked macrophages but were CD45- and CD68-negative. Besides, similar to ordinary follicular cells, RSCs were CK7-positive (**Figure 3a, 3c**), CK8-positive and CK20-negative. Surprisingly, these cells were negative for epCAM (**Figure 3b, 3d**).

In addition, RSCs were calcitonin-negative and showed poor or no reaction with anti-thyroglobulin

antibodies (**Figure 4a, 4c**). RSCs and other PTC epithelial cells were similar in their periodic acid Schiff positivity (**Figure 4b, 4d**).

Pleomorphic cells of another phenotype observed in FNA smears of PTC featured intracellular maculae (macula cells) (**Figure 1b**). This structure, mostly rounded and 4.5-18.0 μm in diameter, was located centrally, sometimes deforming the shape of a

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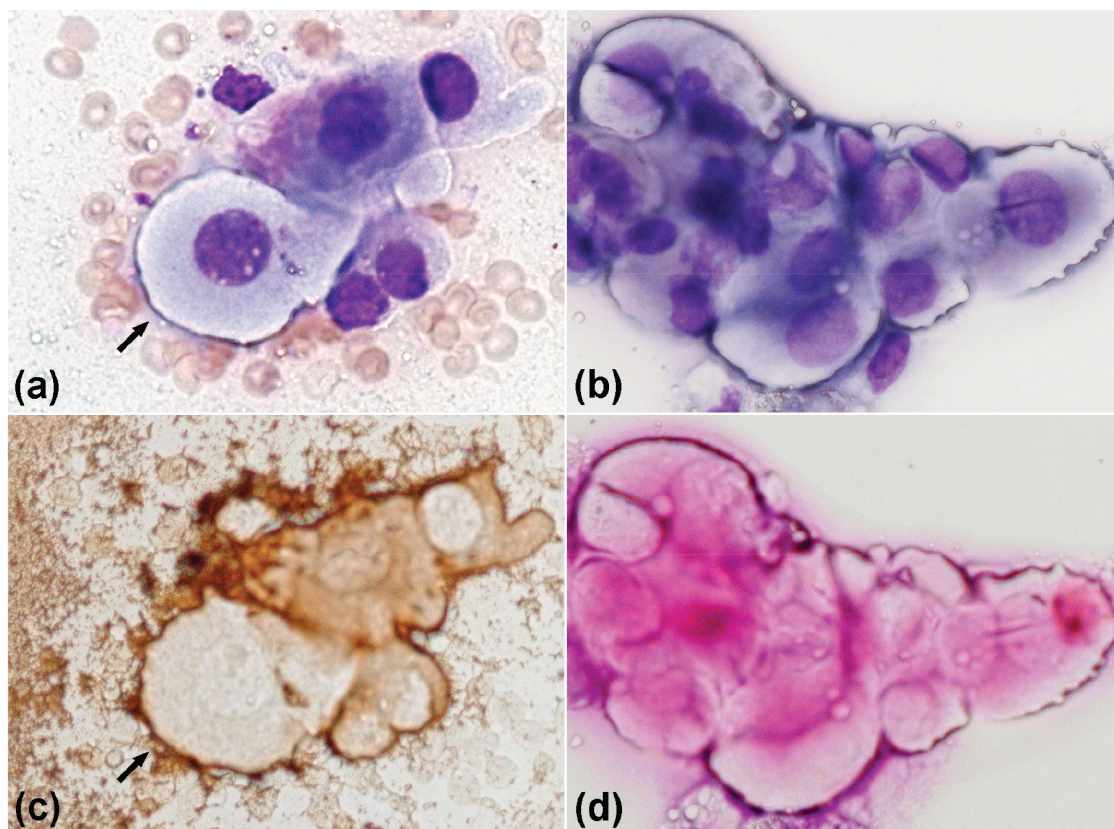


Fig. 4. 'Refractive surface cells' in FNA smears of PTC (a-d; $\times 1000$). Their cytoplasm is thyroglobulin-negative (arrow) (c) and periodic acid Schiff reaction shows no excessive accumulation of polysaccharides (d).

nucleus. Having fuzzy boundaries it was composed of a non-uniform, fine granular basophilic material stained blue by Giemsa dye. Macula cells (MCs) were arranged singly or in clusters, being either isolated or attached to epithelial sheets. MCs were more than double the size of other PTC epithelial cells, i.e., $773.1 \pm 25.0 \mu\text{m}^2$ ($p < 0.01$) (**Figure 2b**).

The cytoplasm of MCs was mildly basophilic, containing occasional inclusions and small vacuoli. In nuclear shape and chromatin structure MCs were similar to other PTC epithelial cells, and some of the former contained intranuclear pseudoinclusions. The latter were found in 67.4% (29/43) of MCs-containing PTC. MCs' nuclear size equaled $156.4 \pm 3.7 \mu\text{m}^2$ being statistically larger than that of other PTC epithelial cells ($p < 0.01$). Binucleate MCs were observed sporadically and some of these cells contained micronuclei.

Despite resembling macrophages visually (**Figure 5 a-d**), MCs were CD45- and CD68-negative. But similar to RSCs, they displayed positivity for CK7 and CK8 (**Figure 5c**) as well as CK20-negativity. The positive reaction for CK7 and CK8 was

often more intensive in the macular region. EpCAM was expressed by MCs in a very specific way. Normally, this molecule is found on the cell surface and in the cytoplasm of epithelial cells but MCs express epCAM only in laminar structures sometimes observed between contacting surfaces of these cells (**Figure 5d**).

MCs were calcitonin-negative and thyroglobulin-positive (**Figure 6a, 6c**), the latter reaction being poor or absent in the macular region. RNase digestion demonstrated that its basophilia was RNA-related (**Figure 6b, 6d**). Occasional mitoses seen in MCs showed they are not degenerative cellular forms.

We found RSCs in 28.2% (79/280) of PTC cases analyzed. In RSCs-containing lesions their share was up to 1.0% of all PTC epithelial cells, though in one case it was as high as 38%. In contrast, MCs were observed more frequently, i.e., in 48.9% (137/280) of analyzed PTC cases. In MCs-containing tumors MCs usually made up to 1% of epithelial cells on a FNA smear totaling up to 30.0% in some cases. We never found RSCs and/or MCs

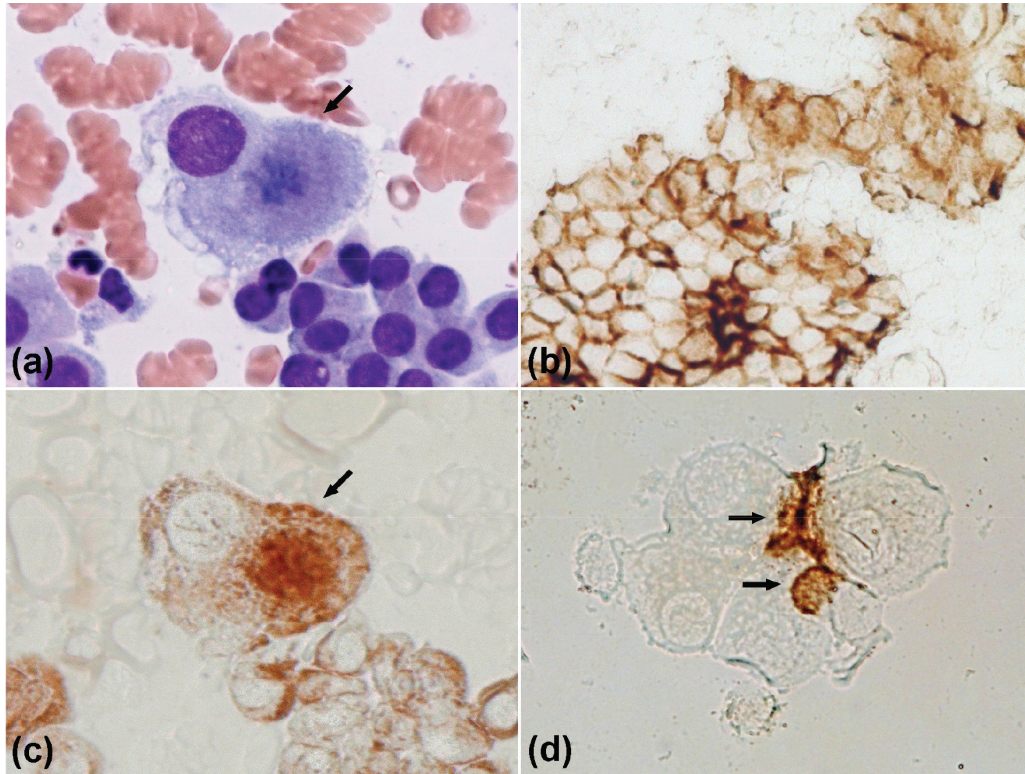


Fig. 5. 'Macula cells' in FNA smears of PTC (a-d). CK8-positivity indicates the epithelial origin of these cells (arrow) (c; $\times 1000$). In contrast to other PTC epithelial cells (b; $\times 700$), they demonstrate a peculiar pattern of epCAM positivity (arrows) (d; $\times 1000$).

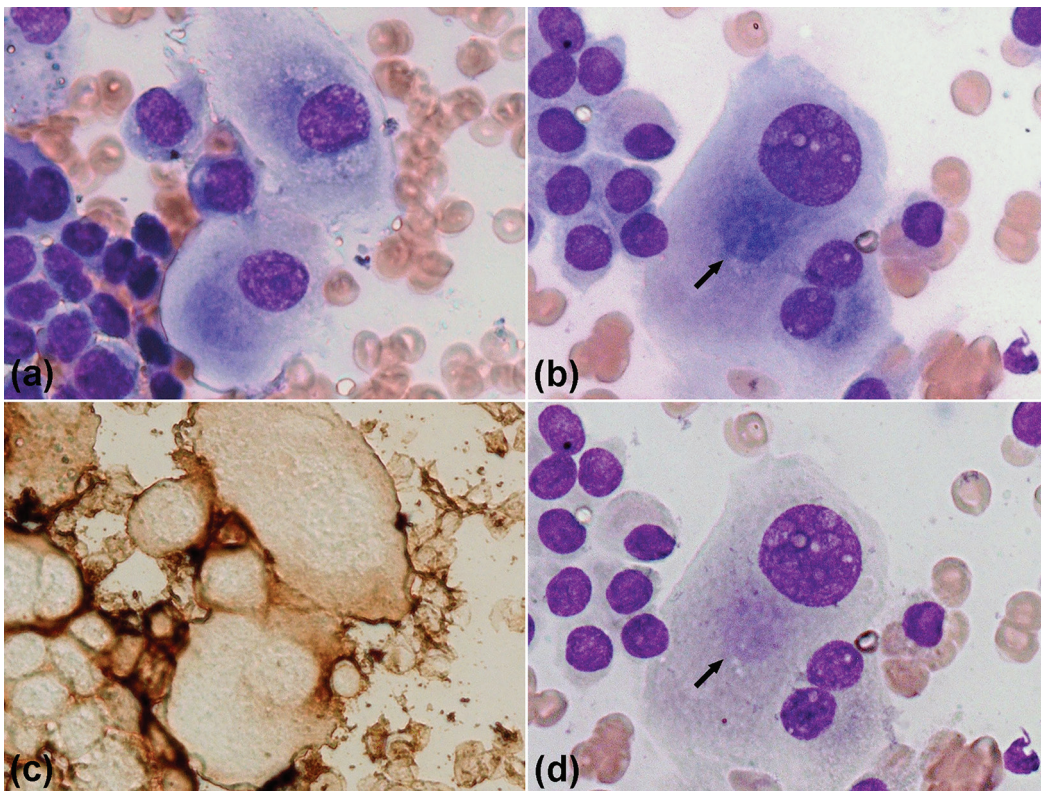


Fig. 6. "Macula cells" in FNA smears of PTC (a-d; $\times 1000$). Their cytoplasm is moderately thyroglobulin-positive (c). RNAse digestion shows that the macula contains RNA (arrow) (b – before, d – after the digestion).

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in FNA smears of nodular goiter (0/620), follicular thyroid adenoma (0/332), Hashimoto's thyroiditis (0/54), oncocytic cell lesion (0/50), follicular thyroid carcinoma (0/22) or medullary thyroid carcinoma (0/42).

Enlarged pleomorphic cells of both phenotypes express CK7 and CK8 presuming their epithelial origin. They are also negative for CK20 and calcitonin. Cells of the first phenotype, i.e., RSCs, are epCAM-negative producing little or no thyroglobulin. The latter feature may be explained by earlier evidence of downregulated and patchy thyroglobulin expression in PTC [10, 11]. RSCs lack features either of oncocytic cells, i.e., large nucleus and oxyphilic cytoplasm, or parafollicular cells, i.e., calcitonin-positivity. Thus, combined features of RSCs as well as their occasional integration into epithelial sheets indicate their epithelial and, most likely, follicular cell origin.

It remains to be investigated why the cytoplasm of RSCs turns 'clear'. Possible explanations include the accumulation of mitochondria-derived vesicles, mucin-depositing vacuoles, glycogen, thyroglobulin, or lipids reported earlier for PTC cells [12, 13]. However, the thyroglobulin-negativity, usual levels of periodic acid Schiff positivity and rare vacuoli or lipid inclusions observed in the cytoplasm of RSCs allow to exclude from consideration at least some factors mentioned above.

An intracellular macula is the most distinctive feature of another group of enlarged pleomorphic cells found in FNA smears of PTC, i.e., MCs. As evidenced by their immunophenotype, except epCAM expression, MCs are of follicular cell origin. The question whether RSCs and MCs are related cell forms is open to discussion.

It was found that RSCs and MCs could mimic macrophages. Atypical macrophage-like so-called 'histiocytoid' cells were described earlier in PTC [14]. A detailed comparison between RSCs and MCs, on the one hand, and 'histiocytoid' cells, on the other, may prove difficult because the immunophenotype of the latter is poorly studied. Though, a number of morphologic features, namely light-refracting cell borders of RSCs, basophilic macula of MCs, and small numbers of vacuoli typical of both RSCs and MCs make them different from 'histiocytoid' cells.

The sensitivity of RSCs and MCs as PTC markers was earlier found to be 28.4% and 48.9%, respectively [15]. It is lower than that of Hector Battifora

mesothelial antigen-1 [16-19], CK19 [17-19] and galectin-3 [17, 19, 20]. However, being inferior to other PTC markers in sensitivity, RSCs and MCs are superior in specificity. Notwithstanding the fact that intranuclear pseudoinclusions occur in 80-85% of PTC cases, they are unspecific being occasionally observed in papillary hyperplasia, Hashimoto's thyroiditis, hyalinizing trabecular tumor and oncocytic cell neoplasm [2, 20]. A meta-analysis demonstrated that Hector Battifora mesothelial antigen-1, CK19 and galectin-3 are 73-83% specific in the detection of PTC [20]. All this is in sharp contrast to 100% specificity shown either by RSCs or MCs.

Earlier, we found that fragments of transformed follicular epithelium observed in FNA smears of PTC display two distinct cell patterns never seen within the same epithelial cell sheet [9]. The first pattern, a regular one, is formed by evenly spaced cells and can be found frequently. The second, irregular cell pattern has the appearance of cohesive cell clusters formed by highly pleomorphic cells and can be seen occasionally. Our findings suggest that RSCs and MCs are, at least in part, responsible for the specific appearance of the second pattern.

This study was performed using air-dried, conventional Giemsa stained smears. In many aspects they are different from liquid-based preparations gaining popularity in the last decade, especially by the amount of the background material [21]. In conventional smears, cells are embedded into the homogeneous layer of a dried mixture of blood plasma and colloid. In this case refractive-index differences are small between the cytoplasm of a follicular cell and the background material, making cell borders barely discernible and sometimes invisible. This focuses the attention of a cytologist on RSCs ensuring their easier identification. One may assume that these cells look different in liquid-based preparations. Additional research is required to evaluate how they perform in the identification of RSC or MC.

Our study has several limitations. First, it is not clear whether the identified PTC cell phenotypes relate to independent clones of tumor cells. We can only assume, for now, that such relation is possible, on the ground of the specific morphology, immunophenotype and capacity of RSCs and MC to form cohesive clusters.

Second, the invasive/metastatic potential of RSCs and MCs and, thus, their value to predict the aggressive behavior of PTC remains unclear. An

impaired epCAM expression typical of RSCs and MCs may indicate the involvement of these cells in invasive processes. In this context, it would be useful to compare the occurrence of RSCs and MCs in PTC cases grouped by levels of invasive/metastatic growth, as well as to analyze the proliferative activity of these cells.

Third, as RSCs and MCs were identified and studied in FNA smears we do not know precisely what histological structures of a PTC (papillae, solid areas etc) they originate from. Such information would assist in exploring the diagnostic potential of RSCs and MCs. Further research is required to clarify the issues mentioned above.

Conclusions

Our findings suggest that RSCs and MCs occur in FNA smears of PTC but never in smears of benign or non-PTC malignant lesions of the thyroid, having the epithelial origin. RSCs and MCs demonstrate a limited sensitivity, but both show 100% specificity in the detection of PTC. The unusual morphology of these cells permits their easy identification without time-consuming immunocytochemistry. The use of RSCs or MCs as morphologic markers complementary to the panel of other PTC markers may improve the reliability of PTC detection in FNA cytology, especially in Giemsa-stained smears, in which nuclear features are less discernible.

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Abbreviations

- CK** – cytokeratin
EpCAM – epithelial cell adhesion molecule
FNA – fine needle aspiration biopsy
MC – macula cell
PTC – papillary thyroid carcinoma
RSC – refractive surface cell

Acknowledgments

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Оригінальні дослідження

Два незвичайні фенотипи епітеліальних клітин дозволяють виявляти папілярну карциному в тонкогolkових аспіратах щитоподібної залози

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Резюме. Тонкогolkова аспіраційна біопсія (ТАБ) є найбільш часто використовуваним методом діагностики папілярної карциноми (ПКЩ) та інших уражень щитоподібної залози. Однак цитоморфологічні маркери ПКЩ, що застосовуються зараз у діагностичній ТАБ цитології, є недостатньо специфічними. Вони спостерігаються як при злoякісних, так і при доброякісних ураженнях, експресуються клітинами не кожного підтипу ПКЩ та різною часткою популяції клітин пухлини. **Мета** полягала в дослідженні нових морфологічних маркерів ПКЩ, що можуть бути застосовані при дослідженні мазків ТАБ матеріалу щитоподібної залози. **Матеріал і методи.** Матеріал дослідження включав ТАБ мазки вузлового зобу (n=620), фолікулярної аденоми щитоподібної залози (n=332), тиреоїдиту Хашимото (n=54), онкоцитарних пухлин (n=50), фолікулярної карциноми щитоподібної залози (n=22), медулярної карциноми щитоподібної залози (n=42) та ПКЩ (n=280). Були досліджені ТАБ мазки від пацієнтів обох статей (1164 жінки та 236 чоловіків) віком від 17 до 87 років. Для їх аналізу були застосовані цитохімія, імуноцитохімія та комп'ютерна морфометрія. **Результати.** В ТАБ мазках ПКЩ були ідентифіковані епітеліальні клітини двох незвичайних фенотипів, яких ніколи не виявляли в мазках доброякісних і не-ПКЩ злoякісних уражень щитоподібної залози. Імуноцитохімічне виявлення цитокератинів (ЦК) вказало на епітеліальне походження цих збільшених і плеоморфних клітин. Основні особливості першого фенотипу включали світлу цитоплазму, чітко окреслені контури клітин, що сильно заломлювали світло, низьку або негативну реакцію на тиреоглобулін та негативну реакцію на ерСАМ. Клітини другого фенотипу мали макулу, що містила РНК, демонстрували позитивну реакцію на тиреоглобулін і специфічний патерн експресії ерСАМ. Питання, чи є клітини двох ідентифікованих фенотипів спорідненими формами клітин, є відкритим для дискусії. **Висновки.** Виявлені клітини зустрічаються лише в ПКЩ і, таким чином, можуть бути використані як високоспецифічні морфологічні маркери, що потенційно здатні підвищити надійність виявлення ПКЩ при дослідженні звичайних ТАБ мазків щитоподібної залози, забарвлених за методом Гімза. Незвичайна морфологія цих клітин дозволяє легко ідентифікувати їх без трудомісткої імуноцитохімії.

Ключові слова: діагностична цитологія, тонкогolkова аспірація, папілярна карцинома щитоподібної залози.

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