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ASSESSMENT OF LABILE ZINC AND COPPER POOLS IN THE PROSTATE TUMOR MICROENVIRONMENT: A COMPARATIVE ANALYSIS OF METHODOLOGICAL APPROACHES

Prostate cancer (PCa) is characterized by complex disturbances in cellular metabolism and remodeling of the tumor microenvironment, in which trace elements may play an important role. Zinc and copper are involved in the regulation of numerous enzymatic, metabolic, and signaling processes, and alterations in their intratissue homeostasis may accompany malignant transformation. Of particular interest are the labile pools of these metals, which reflect the fraction available for biochemical interactions and may serve as sensitive indicators of metabolic changes in tumor tissue. ***Aim:*** *to comparatively assess the levels of labile zinc and copper pools in the tissue of benign and malignant prostate neoplasms and to determine the methodological features of their fluorescent analysis.* ***Object and Methods:*** *the study was performed on tissue samples from 10 patients with benign prostatic hyperplasia (BPH) and 15 patients with PCa. The fluorescent probes ZnAF-2F and Coppersensor 3 were used to determine labile forms of zinc and copper, followed by analysis using a microplate reader and digital fluorescence microscopy of FFPE sections.* ***Results:*** *according to the results obtained from unfixed PCa tissue, the level of labile zinc was 28.8% lower, whereas the copper level did not differ statistically from that in BPH samples. In the analysis of FFPE sections, the level of labile zinc in PCa tissue was 2.8-fold lower, whereas the copper level was 1.2-fold higher compared with BPH.* ***Conclusion:*** *the obtained results demonstrate a common trend toward a decrease in the labile zinc pool and preservation or elevation of copper levels during malignant transformation. Combining integral fluorometric and spatial digital analyses may provide a more comprehensive assessment of disturbances in metal homeostasis in PCa; however, the limitations of FFPE material should be taken into account.*

Keywords: *prostate cancer, benign prostatic hyperplasia, zinc, copper.*

Prostate cancer (PCa) is one of the most common malignancies in men and remains an important medical and biological problem [1]. Despite significant advances in the diagnosis and treatment of this cancer, the factors determining the development of an aggressive phenotype remain unclear. Among the factors that substantially affect the functional state of tumor cells and their microenvironment, metals occupy a special place as trace elements essential for the functioning of a wide range of enzymes, transcription factors, and signaling systems. Disturbances in their intracellular and tissue homeostasis may significantly alter metabolic processes, redox status, and the proliferative and invasive activity of transformed cells [2–4].

Among the metals that are critical for the course and progression of PCa are zinc and copper, which play key roles in the regulation of apoptosis, oxidative stress, and metabolic reprogramming of malignant cells

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[5–7]. Zinc is a cofactor for more than 300 enzymes and an important component of transcription factors, superoxide dismutase, and various proteins involved in DNA repair [6]. The prostate has a unique biochemical profile that markedly distinguishes it from most other soft tissues of the human body. A hallmark of healthy prostatic epithelium is its exceptionally high zinc content, with zinc concentrations being substantially higher than those in other organs. This natural phenomenon is not merely the storage of a trace element but represents a fundamental basis of the specialized metabolic function of secretory cells, which is directed toward the excessive synthesis and accumulation of citrate [8, 9].

One of the most pathognomonic and pathophysiologically significant metabolic features of the malignant transformation of the glandular epithelium of the prostate is the loss of the cells' ability to accumulate zinc. A decrease in intracellular zinc levels may occur as early as the initial stages of carcinogenesis, before the appearance of pronounced histopathological changes, and may continue during disease progression. One of the key mechanisms underlying this phenomenon is impaired expression and function of zinc transporters of the ZIP (SLC39) and ZnT (SLC30) families, which regulate the uptake of Zn^{2+} into cells, its intracellular distribution, and its export. In particular, reduced expression of ZIP1 and other transporters capable of promoting zinc accumulation in prostatic epithelial cells is considered an important component of the molecular alterations associated with PCa development. At the same time, the biological role of zinc in malignant transformation is not limited to its involvement in citrate metabolism. Zinc is a structural and functional component of hundreds of proteins and enzymes and participates in the regulation of transcription, DNA repair, cell signaling, and apoptosis. Therefore, changes in its intracellular availability may affect numerous processes that determine tumor cell behavior [10, 11].

Another critically important trace element involved in cellular respiration, antioxidant defense, angiogenesis, and the function of enzymes responsible for extracellular matrix (ECM) remodeling is copper. Unlike zinc, the progressive depletion of which characterizes the early stages of oncogenic transformation of the glandular epithelium, the biological significance of copper in malignant neoplasms has a fundamentally opposite direction. The role of this trace element in oncogenesis is associated with its ability to act as a potent cofactor and driver of processes required for enhanced tumor growth, neoangiogenesis, and progression. Systemic and local disturbances in copper homeostasis have been described in a number of malignant neoplasms and are accompanied by its active accumulation in neoplastic tissue [2, 12]. Copper transport and intracellular availability are regulated, in particular, by the CTR1 transporter, ATP7A/ATP7B, and a number of intracellular chaperones [13].

Copper is of particular importance due to its role in ECM remodeling. Copper is required for the function of lysyl oxidases (LOX and LOXL1–4), enzymes that catalyze the oxidative cross-linking of collagen and elastin. Dysregulation of LOX-dependent matrix remodeling may alter its mechanical properties, promote the formation of a tumor-supportive microenvironment, and create conditions favorable for invasion and metastasis. Thus, changes in copper availability may be associated not only with the metabolic characteristics of tumor cells but also with remodeling of the stromal component of neoplasms [14, 15].

Given that Zn^{2+} and $Cu(I)/Cu^{2+}$ have distinct biological functions and exist in tissues as several interconnected pools, assessment of their total content does not always reflect the functionally available fraction of the metal. Of particular interest is the assessment of labile metal pools, which may directly participate in intracellular signaling and enzymatic processes. Fluorescent chemical probes are a promising tool for such investigations because they enable highly sensitive detection of metal ions while preserving information about their spatial distribution within tissues [16].

Therefore, comparative assessment of methods for the fluorescent detection of Zn^{2+} and Cu(I) in tissues of benign and malignant prostate neoplasms may be important for establishing methodological approaches to evaluating their homeostasis in PCa. The application of such approaches may potentially characterize differences between benign and malignant tissues not only in terms of metal levels but also in terms of their spatial availability, which may be relevant to understanding metabolic reprogramming, ECM remodeling, and the development of an aggressive PCa phenotype.

MATERIALS AND METHODS

The study was performed on clinical material (tumor tissue) obtained from 10 patients with benign prostatic hyperplasia (BPH) and 15 patients with PCa who underwent examination and treatment at the Center for Modern Urology (Kyiv, Ukraine) in 2023–2026 and provided informed consent for the use of their clinical data for scientific purposes. The age of patients with PCa ranged from 45 to 80 years, with a mean age of 63.0 ± 2.8 years. The mean age of patients with BPH was 66.1 ± 4.5 years. Patients were examined according to the standards for the diagnosis and treatment of patients approved by the Ministry of Health of Ukraine.

The clinical diagnosis was established based on serum prostate-specific antigen (PSA) levels, digital rectal examination, computed tomography of the pelvic organs and/or transrectal ultrasonography of the prostate and abdominal organs, bone scintigraphy, and chest radiography. In all patients, the diagnosis was verified by transrectal ultrasound-guided multifocal

prostate biopsy. Tumor stage was determined according to the International Classification of Tumors (TNM, 8th edition, 2016). Histological sections were stained with hematoxylin and eosin. Histological tumor types were verified by microscopic examination according to the updated fifth edition of the WHO Classification of Urinary and Male Genital Tumours (2022) [17] and the 2014 consensus of the International Society of Urological Pathology (ISUP) [18]. Exclusion criteria were poor tissue preservation, low-quality histological material, and the administration of specific treatment at the time of diagnosis or surgical intervention. The study was approved by the Institutional Review Board and local Ethics Committee and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Determination of Zinc and Copper Content in Prostate Neoplastic Tissue Using a Microplate Reader. Fluorescent probes ZnAF-2F (Sigma-Aldrich, USA) and Coppersensor 3 (Toronto Research Chemicals, Canada) were used to assess zinc and copper ions in prostate tissue, respectively. To prevent the loss of labile metal forms, the samples were immediately frozen in liquid nitrogen. Frozen tissue was mechanically homogenized in a pre-cooled porcelain mortar until a homogeneous powder was obtained, with continuous cooling in liquid nitrogen. To ensure further disruption of cellular structures and extraction of labile ions, the resulting suspension was sonicated on an ice bath for 15 s.

For zinc and copper determination, 100 µL of the corresponding fluorescent probe solutions, ZnAF-2F and CS3, at concentrations of 10 µM and 2 µM, respectively, were added to 2 mg of cryopreserved tissue. The mixtures were vortexed for 30 s and incubated in the dark at 37°C for 40 min. After incubation, the suspensions were centrifuged at 13,000×g for 15 min at 4°C. The resulting supernatants were transferred to a black, flat-bottom 96-well microplate (Thermo Fisher Scientific, USA) at 100 µL per well. Working solutions of the corresponding fluorescent probes without biological material were used as blanks. Empty wells were used to determine the background fluorescence of the plate. Fluorescence intensity was measured using a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. The obtained values were used to assess the relative zinc and copper content in the studied samples. Measurements were performed in triplicate for each sample.

Determination of the Relative Levels of Labile Copper and Zinc Pools in Prostate Neoplastic Tissue Using Digital Microscopy. Formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples were used in the study. Tissue sections 5 µm thick were obtained using an AMR 400 microtome (Amos Scientific, Melbourne, Australia). Before fluorescent staining, the FFPE samples were deparaffinized in xylene, washed in 96% ethanol, and rehydrated in distilled water.

Fluorescent probes Coppersensor 3 and ZnAF-2F were used to assess the relative levels of labile Cu(I) and Zn^{2+} pools in prostate neoplastic tissue, respectively. Working solutions of CS3 at a concentration of 2 µM and ZnAF-2F at a concentration of 10 µM were used for staining. Incubation was performed for 30 min at 37°C. Staining was performed using the dropwise method by applying 30 µL of the corresponding fluorescent probe solution to the surface of each tissue section. To reduce nonspecific binding of the probes to the tissue, the sections were washed with PBS cooled to 4°C. The tissue was counterstained with DAPI (4′,6-diamidino-2-phenylindole) (Thermo Fisher Scientific, USA) for visualization of cell nuclei. Tissue autofluorescence was assessed using a negative control, in which 30 µL of PBS was applied to separate sections instead of the fluorescent probe solution.

The fluorescent signal was recorded using an Axio Scope A1 microscope (ZEISS GmbH, Germany) equipped with an HXP 120 fluorescence illuminator and a ZEISS Axiocam 820 color digital camera. Fluorescence was excited using a high-efficiency Carl Zeiss Filter Set 38 HE, featuring a blue excitation bandpass filter (BP 470/40), and the emission was recorded through a green emission bandpass filter (BP 525/50). The exposure times for imaging tissue stained with ZnAF-2F and CS3 were 500 and 1000 ms, respectively. At least 15 randomly selected fields of view were analyzed for each tissue sample. Fluorescence intensity was determined in micrographs using ImageJ v. 1.42 (https://imagej.nih.gov/ij/). The mean fluorescence intensity (MFI) in samples of benign and malignant prostate neoplasms was calculated using the following formula:

$$\mathrm{MFI} = \mathrm{Integrated\ Density}_{\mathrm{tissue}} - \left[\frac{\mathrm{Integrated\ Density}_{\mathrm{background}}}{\mathrm{Area}_{\mathrm{background}}} \times \mathrm{Area}_{\mathrm{tissue}}\right].$$

To determine the absolute fluorescence values, tissue autofluorescence was subtracted from the median values of the experimental samples.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism v. 10 (GraphPad Software Inc., San Diego, CA, USA). Quantitative variables between two independent groups were compared using Student's *t*-test for parametric data or the Mann–Whitney U test for non-parametric data. Results were graphically presented as box-and-whisker plots, with the central line representing the median and the lower and upper boundaries of the box representing the first and third quartiles, respectively. The whiskers extending from the boxes indicate the minimum and maximum values in the studied groups, while black dots represent outliers. Differences between groups were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

As shown in *Fig. 1a*, the mean fluorescence intensity of the ZnAF-2F probe in unfixed BPH tissue was 11,985.0 ± 286.8 arbitrary units (a.u.), which was 28.8% higher ($p = 0.0242$) than that in PCa tissue (9,307.3 ± 718.4 a.u.). The mean fluorescence intensity of the CS3 probe was 5,490.9 ± 388.0 a.u. in PCa tissue and 5,347.3 ± 402.2 a.u. in BPH samples. No statistically significant differences in the levels of the labile copper pool were detected between the studied samples (*Fig. 1b*).

Subsequently, the levels of labile zinc and copper pools in fixed prostate tumor tissue were analyzed using fluorescence microscopy (*Fig. 2*). As shown in *Fig. 1c*, PCa tissue was characterized by a 2.8-fold lower level of labile zinc ($p < 0.0001$) compared with BPH tissue. In contrast, the level of the labile copper pool in PCa tissue was 1.2-fold higher ($p = 0.0400$) than that in BPH tissue (*Fig. 1d*).

Thus, our results clearly demonstrate that the absolute values and fold changes in the levels of labile zinc and copper pools depended substantially on the methodological approach used. In particular, considerable discrepancies were observed between the analysis of unfixed homogenized lysates using a microplate reader and spatial measurements performed on fixed histological sections (FFPE) by digital microscopy. Nevertheless, despite differences in absolute values and effect sizes, both methods demonstrated a consistent biological trend — a marked decrease in the labile zinc pool and preservation or elevation of copper levels during malignant transformation.

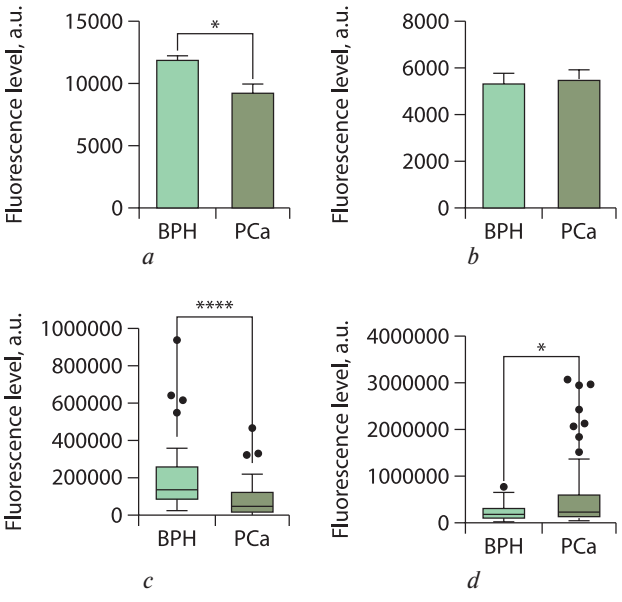


Fig. 1. Relative levels of labile zinc (*a* and *c*) and copper (*b* and *d*) pools in tissue from benign and malignant prostate neoplasms, as determined by microplate analysis (*a* and *b*) and quantitative digital microscopy (*c* and *d*)

Notes: * — statistically significant difference compared with BPH ($p < 0.05$); **** — statistically significant difference compared with BPH ($p < 0.0001$).

It should be emphasized that the labile pools of zinc and copper do not represent the absolute total content of these metals in tissue. Traditionally, the localization of zinc in archival histological material or paraffin blocks has been visualized using histochemical methods based on the formation of colored insoluble precipitates, such as the Timm sulfide-silver method [19]. Classical histochemical methods based on staining with rubeanic acid, rhodanine, and orcein are also successfully used to detect copper in tissues [20]. However, the use of fluorescent sensors opens up qualitatively new possibilities for assessing metal levels, particularly their labile pools, by providing high sensitivity and enabling their precise localization within cellular compartments and individual components of the tumor microenvironment.

A key advantage of using histological sections for the visualization and quantitative assessment of labile zinc and copper pools, compared with bulk approaches based on whole-tissue homogenized lysates, is the high spatial resolution. This analytical approach enables precise topological analysis directly within the neoplastic parenchyma.

The use of tissue sections allows detailed differentiation of the internal architecture of the neoplasm, which is not possible with integrated analysis of tissue homogenates. This method enables clear discrimination between the tumor parenchyma (malignant epithelium) and stromal components, as well as the exclusion of structural artifacts, glandular lumina, vascular spaces, and fibrotic areas from fluorescence intensity measurements. Such selection of regions of interest is particularly important because numerous heterogeneous components of prostate tissue, including structural proteins (collagen and elastin), cofactors (NAD(P)H and flavins), organelles (mitochondria and lysosomes), and erythrocytes, exhibit substantial autofluorescence. This endogenous background signal can significantly distort fluorimetric measurements if it is not spatially separated [21–23].

An additional fundamental advantage of spatial analysis using tissue sections is the possibility of parallel morphological assessment. Integration of specific fluorescent sensing of labile metal pools with conventional cytomorphological methods, such as fluorescent nuclear counterstaining with DAPI or routine hematoxylin and eosin staining, provides a unique opportunity to detect subtle local gradients of metal distribution.

At the same time, the use of sections prepared from FFPE tissue is associated with substantial limitations. In particular, fixation in 10% neutral buffered formalin cross-links free amino acid residues, including cysteine, histidine, and glutamic acid residues; however, a substantial proportion of the labile zinc pool does not form strong covalent bonds with proteins.

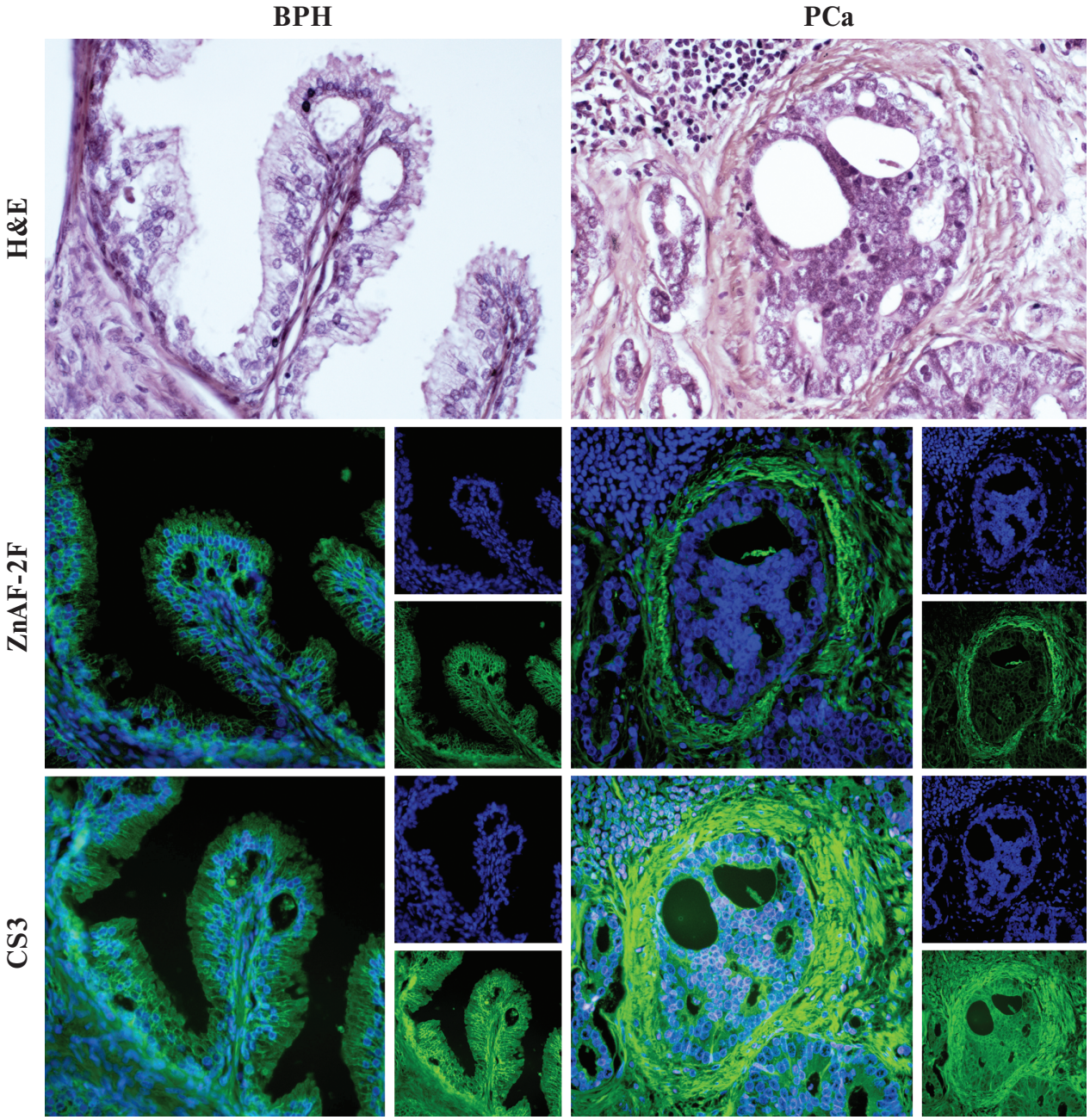


Fig. 2. Visualization of labile zinc and copper pools (green fluorescence) in benign prostatic hyperplasia (BPH) and prostate cancer (PCa) tissues using the fluorescent probes ZnAF-2F and CS3, respectively. Nuclei were counterstained with DAPI (blue fluorescence). Fluorescence microscopy, ×400 magnification. Representative histological sections stained with hematoxylin and eosin (H&E) are shown for comparison; light microscopy, ×400

During subsequent tissue processing through increasing concentrations of alcohol and xylene, free and weakly bound metal ions may be extracted from tissue compartments. In addition, during formalin fixation and subsequent processing through alcohol and xylene, some metals may form stable, non-reactive chelates with denatured proteins [24, 25].

Thus, fluorescence analysis of FFPE material likely reflects only the residual fraction of metals that withstands the aggressive chemical processing and remains preserved in stable coordination complexes or precipitated forms. Therefore, from a methodological perspective, the optimal approach for assessing the in vivo distribution of trace elements is the use of cryostat sections prepared from frozen tissue. However, in routine clinical and laboratory practice, this approach is often substantially limited by the lack of cryogenic equipment or the inability to rapidly freeze surgical specimens *in situ*.

Standardization of preanalytical conditions is therefore particularly important, including the time to fixation or freezing, the type and duration of fixation, tissue-processing conditions, section thickness, and the parameters of fluorescence excitation and detection.

Considering the advantages and limitations of each platform, it becomes evident that the two approaches used in this study — analysis of homogenized samples of unfixed tissue and fluorescence analysis of histological sections — are complementary. Rather than competing with each other, they form a two-level analytical system that can minimize the methodological biases inherent to each approach. In our view, the rational combined use of these methods provides a more comprehensive representation of the pathophysiological alterations associated with metal homeostasis in tumor tissue.

Specifically, microplate fluorimetric analysis of tissue lysates provides an integrated assessment of the fluorescence signal across the entire volume of the analyzed sample. This platform is high-throughput, standardized, and convenient for the rapid comparison of large amounts of biological material, including large patient cohorts or series of experimental samples. It also minimizes the impact of local heterogeneity, whereby a randomly selected section may not adequately represent the overall trend within the tumor.

In contrast, digital fluorescence microscopy of tissue sections serves as a precise spatial verification tool. It preserves information on the spatial distribution of trace elements within the analyzed tissue specimen. Owing to its high spatial resolution, this approach enables dynamic changes in labile metal pools to be associated with specific cellular compartments, such as the cytoplasm and mitochondria, as well as distinct structures of the tumor microenvironment, including areas of neoangiogenesis or stromal desmoplasia.

The combination of these methods may provide a more comprehensive understanding of disturbances in metal homeostasis during malignant transformation. Integration of macro- and microanalytical approaches can overcome the methodological limitations of each technique individually, transforming quantitative measurements into a more comprehensive pathophysiological interpretation of the tumor process. From a biological perspective, the observed trend toward a decrease in the labile zinc pool and preservation or elevation of labile copper levels may reflect profound alterations in metal-dependent processes within prostate tumor tissue [5, 6, 12].

CONCLUSION

Alterations in labile zinc and copper pools may represent an important component of tumor microenvironment remodeling in PCa, while understanding the dynamics of these trace elements may provide new insights into the pathophysiological mechanisms underlying oncogenesis. At the same time, their interpretation requires consideration of methodological characteristics, the limitations of fixed tissue material, and the differences between labile and total metal pools.

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ОЦІНКА ЛАБІЛЬНИХ ПУЛІВ ЦИНКУ ТА МІДІ В ПУХЛИННОМУ МІКРООТОЧЕННІ ПЕРЕДМІХУРОВОЇ ЗАЛОЗИ: ПОРІВНЯЛЬНИЙ АНАЛІЗ МЕТОДИЧНИХ ПІДХОДІВ

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Резюме. *Рак передміхурової залози (РПЗ) характеризується складними порушеннями клітинного метаболізму та ремоделюванням пухлинного мікрооточення, у яких важливу роль можуть відігравати мікроелементи. Цинк і мідь беруть участь у регуляції численних ферментативних, метаболічних та сигнальних процесів, а зміна їхнього внутрішньотканинного гомеостазу може супроводжувати злоякісну трансформацію. Особливий інтерес становлять лабільні пули цих металів, які відображають доступну для біохімічних взаємодій фракцію та можуть бути чутливими індикаторами метаболічних змін у пухлинній тканині.* ***Мета:*** *провести порівняльну оцінку рівнів лабільних пулів цинку та міді у тканині доброякісних і злоякісних новоутворень передміхурової залози та визначити методичні особливості їх флуоресцентного аналізу.* ***Об'єкт і методи:*** *дослідження виконане на зразках тканини 10 хворих на доброякісну гіперплазію передміхурової залози (ДГПЗ) та 15 хворих на РПЗ. Для визначення лабільних форм цинку та міді використано флуоресцентні зонди ZnAF-2F і Coppersensor 3 з подальшим аналізом за допомогою мікропланшетного рідера та цифрової флуоресцентної мікроскопії FFPE-зрізів.* ***Результати:*** *згідно з результатами дослідження нефіксованої тканини РПЗ рівень лабільного цинку був на 28,8% нижчим, тоді як рівень міді статистично не відрізнявся від аналогічного у зразках ДГПЗ. При дослідженні FFPE-зрізів рівень лабільного цинку у тканині РПЗ був у 2,8 раза нижчим, а міді — у 1,2 раза вищим порівняно з ДГПЗ.* ***Висновки:*** *отримані результати демонструють спільну тенденцію до зниження лабільного пулу цинку та збереження або підвищення рівня міді при малігнізації. Поєднання інтегрального флуориметричного та просторового цифрового аналізу може забезпечити комплекснішу оцінку порушень гомеостазу металів при РПЗ, однак потребує врахування обмежень FFPE-матеріалу.*

Ключові слова: рак передміхурової залози, доброякісна гіперплазія передміхурової залози, цинк, мідь.

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