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EVALUATION OF DIAGNOSTIC ALGORITHM BASED ON COLLAGEN ORGANIZATION PARAMETERS FOR BREAST TUMORS

N. Lukianova, T. Zadvornyi, O. Mushii, T. Pyatchanina, V. Chekhun*
R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, 03022 Kyiv, Ukraine

The changes in the quantitative parameters and spatial structure of collagen are considered a key diagnostic and prognostic factor associated with the development of many malignant neoplasms, including breast cancer (BCa). *The aim* of the work was to develop and test an algorithm for the assessment of collagen organization parameters as informative attributes associated with BCa for developing technology of machine learning and building an intelligent system of cancer diagnostics. *Materials and Methods*: Tumor tissue samples of 5 patients with breast fibroadenomas and 20 patients with stage I–II BCa were studied. Collagen was identified histochemically by Mallory method. Photomicrographs of the studied preparations were obtained using a digital microscopy complex AxioScope A1. Morphometric studies were performed using the software CurveAlign v. 4.0. beta and ImageJ. *Results*: The algorithm for determining the quantitative characteristics and spatial organization of the collagen matrix in tumor tissue samples has been developed and tested. We showed that collagen fibers in the BCa tissue are characterized by significantly lower values of length ($p < 0.001$) and width ($p < 0.001$) as well as higher values of straightness ($p < 0.001$) and angle ($p < 0.05$) compared to these in the fibroadenoma tissue. No significant difference was found in the density of collagen fibers in the tissue of benign and malignant neoplasms of the mammary gland. *Conclusion*: The algorithm allows assessing a wide range of parameters of collagen fibers in tumor tissue, including their spatial orientation and mutual arrangement, parametric characteristics and density of the three-dimensional fibrillar network.

Key Words: **collagen, breast cancer, breast fibroadenoma.**

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According to the statistics from the WHO and the International Agency for Research on Cancer, one of the most common types of malignant neoplasms in women in the world is breast cancer (BCa). According to GLOBOCAN data, in 2020, 8.8 million people died from malignant neoplasms worldwide, of which 571,000 cases were deaths from BCa. The highest rates of BCa incidence are recorded in Australia, New Zealand, Europe and North America [1]. In Ukraine, BCa also occupies a leading position in terms of cancer-related morbidity and mortality. According to the data of the National Cancer Registry of Ukraine, in 2021 nearly 13,000 BCa cases were diagnosed, which is more than 13% of the total number of diagnosed cancer cases [2].

Among evidence-based methods of medical and biological research, the generally accepted standard is still the pathomorphological method. However, it is known that the informativeness of biological objects obtained using optical microscopy suffers due to the low contrast of the image of cells and cellular structures, the complexity of the organization of tissue structures, the presence of different groups of cells, artifacts in the field of view, and the significant heterogeneity of the tissue as a background factor. In addition, the effectiveness of the study of morphological, histological and immunohistochemical preparations, etc., largely depends on the level of expertise of the medical staff [3].

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*Correspondence: E-mail: nataluk10@gmail.com
Abbreviations used: BCa – breast cancer; ECM – extracellular matrix; FA – fibroadenoma; SHG – second harmonic generation.

To date, it has been proven that the use of automated systems for processing and analyzing microscopic images of histological preparations significantly improves the quality of differential diagnosis and prognosis of the cancer course. To use bioinformatics methods and approaches for the detection and analysis of microphotograph images, a significant volume of scientific and clinical materials has been accumulated [4, 5]. At the same time, the key pathomorphological parameters associated with the development and progression of neoplasms have not been definitively identified, which is especially relevant in the context of BCa, accounting for the high heterogeneity and variability of its clinical course. It should be noted that despite the fact that areas of tumorous and non-transformed tissues have some characteristic colorimetric and morphological features that allow "normal-tumor" recognition, in many cases they can look very similar and difficult to distinguish both using computer image recognition methods and for experts in pathology laboratories. In addition, algorithms of artificial intelligence, which are used for the analysis of microscopic images, provide high reliability only in the setting of clear verification of quantitative and qualitative indicators of the pathological condition [6].

Numerous studies have established that an important role in the progression of many neoplasms, including BCa, belongs to the tumor microenvironment, which is largely determined by the composition of structural fibrillar proteins, represented by various types of collagen, elastin, and other components of the extracellular matrix (ECM) [7–9]. The main fibrillar component of ECM is collagen. According to the recent data [10], the morphology, representa-

tion and organization of collagen fibers contribute to the formation of tumor microenvironment, which stimulates tumor progression by increasing cell migration and polarization. In addition, the remodeling of collagen fibers is of great importance in determining the survival and growth of disseminated tumor cells, and thus the formation of metastases.

Presently, the changes in the ratio of quantitative indicators and the spatial structure of collagen are considered a key diagnostic and prognostic factor associated with the occurrence and progression of many malignant neoplasms, including BCa [11, 12]. Traditionally, the study of collagen in histological slides of tumor tissue is carried out using histochemical trichrome staining methods (according to van Gieson, Masson, Mallory), which allow differential visualization of muscles, collagen, elastin and reticulin fibers, basement membrane, cartilage and fibrin. The study of localization features and semi-quantitative assessment of the geometric parameters of collagen fibers in tumor tissue can also be carried out using the immunohistochemical method and the *in situ* hybridization method [13]. The limited use of these methods for collagen detection is related to the possible development of the unwanted morphological changes in ECM in course of tissue processing that depends to some extent on the expertise of medical personnel [14, 15]. In addition, the disadvantage of these methods is that their use does not allow to fully assess the spatial organization of collagen, which is especially important for elucidating its functional properties.

Recently, to verify the structural organization of collagen, two-photon microscopy is used, which is based on the use of two modalities: tissue fluorescence excited by two-photon absorption (two-photon fluorescence) and second harmonic generation (SHG) by individual biomolecules. SHG microscopy is a highly specific optical method of direct visualization of extracellular fibrillar collagen realized on the basis of most two-photon fluorescence microscopes. A significant advantage of the method is the absence of phototoxic effects [16–18]. The significant disadvantages of both methods are the high cost of equipment and requirement of highly qualified personnel.

The study of the quantitative characteristics of collagen is based mostly on the analysis of microphotographs using specialized software, including ImageJ, FibrilTool, Orientation J, MATLAB (MathWorks, USA), CytoSpectre, CurveAlign, CT-FIRE, as well as FiberFit [19]. The application of the software provides the necessary tools for pre-processing of the image (extraction and segmentation), as well as algorithms for recognition. Most of these programs are designed to work with photomicrographs stained with hematoxylin and eosin. However, it should be noted that this method of staining histological preparations does not allow differentiating collagen from other tissue components, such as elastin, which can lead to false results during morphometry [13].

Machine learning is also a powerful tool for identifying distinctive features of collagen fibers [20] for an effective classification of images taking into account any of their characteristics by predefined categories. Automated approaches for quantification could potentially improve the accuracy of prognostic variables in clinical pathology practice and expand the number of the examined samples.

The modern automated intelligent classifier systems based on artificial neural networks, cluster analysis, deep learning, fuzzy clustering, etc. that use analysis of microphoto images are developed for cancer diagnostics and assessment of tumor progression. At the same time, from the existing variety of tasks, in which artificial neural networks are currently used, classification, prediction and pattern recognition are among the most relevant and the most difficult tasks of intelligent data analysis [21, 22]. In such systems, the problems of building an initial knowledge base, creating a "training sample" are of primary importance, since the information for building neural network algorithms is presented in a set of examples. During the formation of the knowledge base used for the "training" of the artificial neural network, the determined numerical morphometric parameters in accordance with the descriptive information about the patients and the clinicopathological characteristics of the tumor, which form a set of training data, are used to formalize and transform the data for neural network input and creation of a learning algorithm for a classification model.

Therefore, the arsenal of existing modern methodical approaches to visualization of collagen topology (both traditional histological and advanced ones with the use of specialized microscopy) and computational tools for quantitative assessment of changes in the organization of collagen fibers is quite significant; the variety of combinations of applied approaches depends on the specific tasks of the experiment. Regarding the role of collagen disorganization in malignant neoplasms, the need to optimize the method of correctly determining not only the qualitative parameters of its structural organization, but also the quantitative indicators of its spatial characteristics becomes an urgent task.

Therefore, the aim of the work was to develop and test an algorithm for the assessment of collagen organization parameters as informative attributes associated with the BCa for developing technology of machine learning and building an intelligent system of cancer diagnostics.

MATERIALS AND METHODS

Patients. Tumor tissue samples from 5 patients with breast fibroadenomas (FA) and 20 patients with BCa stage I–II who were treated at the Kyiv City Clinical Oncology Center during 2013–2015 were studied. All patients gave informed consent for the use of the clinical data for scientific purposes. The general clinical

characteristics of these patients were presented in our previous study [23].

Histochemical methods. Determination of collagen in the tissue of benign and malignant neoplasms of the mammary gland was carried out by histochemical methods according to Van Gieson, Masson, Mallory. The serial sections of tumor tissue with a thickness of 5 μm were stained using the dyes "Van Gieson trichrome" (DiaPath, Italy), "Masson trichrome" (DiaPath, Italy) and "Mallory trichrome" (DiaPath, Italy) according to the manufacturer's recommendations.

Morphometric methods. Photomicrographs of the studied preparations were obtained using a digital microscopy complex AxioScope A1 (Carl Zeiss, Germany) using an AxioCam ICc5 camera (Carl Zeiss, Germany), with a magnification of ×260. The morphometric determination of the quantitative and spatial organization of collagen fibers in the photomicrographs of tumor tissue stained with Mallory's trichrome was carried out using programs CurveAlign v. 4.0. beta (https://eliceirilab.org/software/curvealign/) and ImageJ (https://imagej.nih.gov/ij/). In order to determine the alignment of collagen fibers, the CT module of the CurveAlign v 4.0 beta program was used. For a more detailed characterization of the quantitative indicators of the organization of collagen fibers (length, width, straightness, and angle) in the tissue of breast tumors using the CT-FIRE module, pre-processing of the obtained microphotographs was carried out using Adobe Photoshop CC (Adobe Systems Inc., CA, USA). In particular, with the use of a graphic editor, tumor tissue components were segregated according to their coloring, which made it possible to increase the accuracy of the CurveAlign program's determination of collagen structures. The density of collagen in the tissue was determined as the ratio of the number of collagen fibers, measured using the ImageJ program, to their area, obtained when working with the CT-FIRE module.

Statistical analysis of the results was performed using the GraphPad Prism v.8.0 program (GraphPad Software Inc., USA). Comparison of the significance of differences in mean values was performed using the Mann — Whitney U-test. Quartile diagrams ("boxplot") were used to graphically present the results of the study, in which the central line marked the median, the lower and upper limits of the "box" were the first and third quartiles, respectively. The whiskers coming out of the rectangles indicate the minimum and maximum values of the indicators in the studied groups. The p value < 0.05 was considered significant.

RESULTS AND DISCUSSION

At the first stage of the work, for the morphometric analysis of collagen in the tissue of benign and malignant neoplasms of the breast, the most correct method of histochemical staining was selected. The visual examination of the samples of tumor tissue indicated the priority of the staining by Mallory, compared to the methods of van Gieson and Masson. The staining by Mallory guarantees the highest level of selectivity, a sufficient level of contrast to fully convey the architecture of the tissue, and is the most accessible for use in the conditions of a pathology laboratory. The disadvantage of the images of BCa specimens stained by van Gieson is their low contrast in a case of a low collagen level, which makes it difficult to segregate them at the pre-preparation stage of microphoto processing for work in the CT-FIRE module of the CurveAlign program. Moreover, a significant disadvantage of this staining method is the need for control under a microscope, since picrofuchsin, as a differentiating compound, weakens the intensity of staining with hematoxylin. Another significant drawback is the fading of staining due to the loss of staining of fuchsin-philic collagen fibers, which indicates the need for a relatively quick analysis of the preparations. A significant disadvantage of using Masson's staining method is the non-uniformity of the staining of collagen structures in the tissue (they acquire colors from blue to light green), which complicates their segregation on a photomicrograph.

In order to obtain a high-quality expert assessment of the segmentation, contour analysis, texture analysis, detection and classification of microphoto objects, a combination of visual assessment and determination of quantitative patterns by means of digital morphometric analysis has been used.

The analysis of indicators of quantitative and spatial disorganization of collagen fibers in the tissue of benign and malignant neoplasms of the breast was carried out according to the algorithm presented in Fig. 1 (described in detail in Materials and Methods section). It is known that using the CurveAlign package allows obtaining the widest range of characteristics of the fibrillar component of ECM. Analysis in the CT-FIRE mode allows obtaining values of the length and width of the fibers, the degree of their straightness and angle relative to the spatial placement, as well as their number. The classic CT mode, in turn, allows obtaining the value of fiber alignment relative to each other. In order to expand the number of descriptive numerical characteristics of the collagen framework, we also used ImageJ to calculate the area of the collagen network shown in the photomicrograph and estimate its density.

The evaluation of the developed algorithm made it possible to establish certain differences in quantitative indicators of the organization of collagen fibers (length, width, straightness, alignment, and angle) in the tissue of benign and malignant neoplasms of the breast. As shown in Fig. 2, collagen fibers in the BCa tissue were characterized by a significantly smaller length ($p < 0.001$) and width ($p < 0.001$) compared to the similar parameters of the FA tissue. It should be noted that such indicators as the length and width of collagen fibers, obtained during the analysis in the CT-FIRE mode, are usually expressed in pixels and do not depend on the size of the obtained image. However, the magnification (number of pixels/

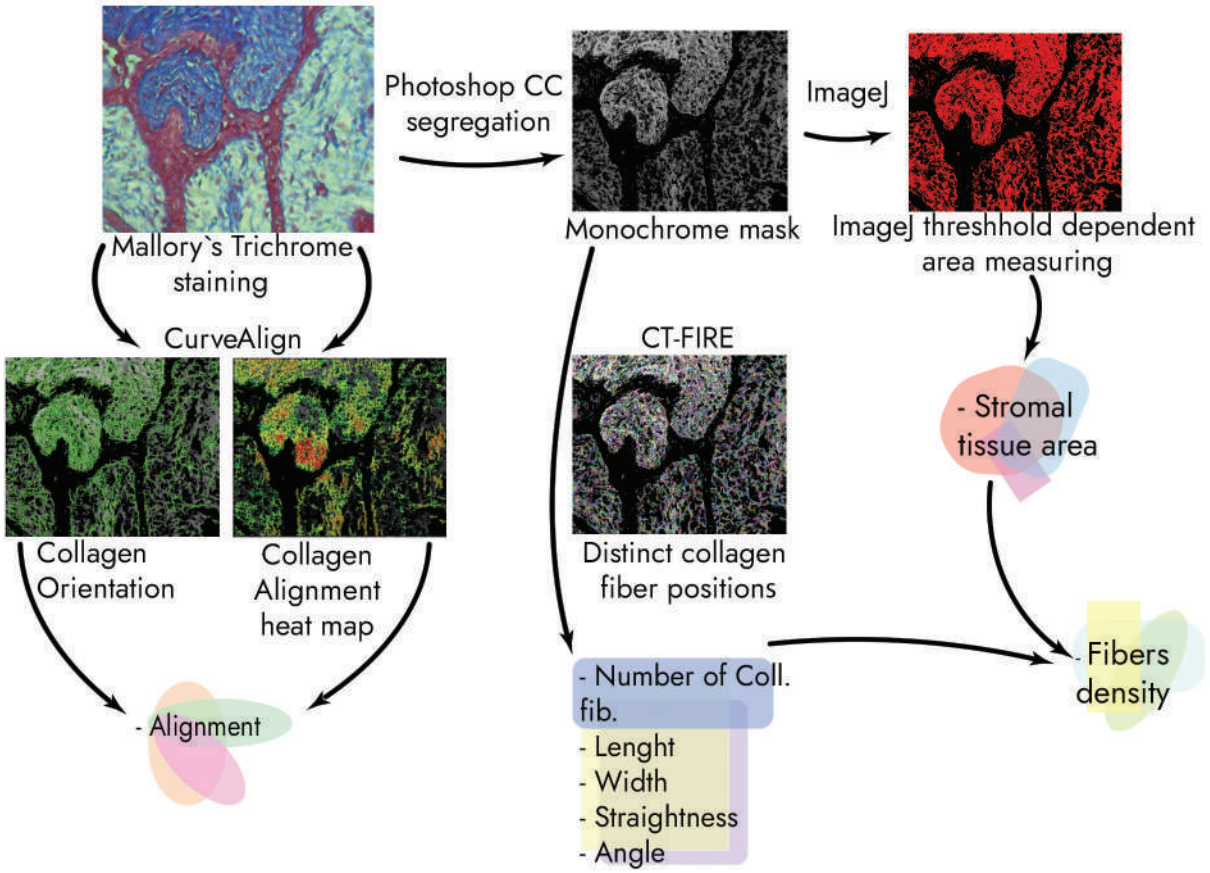

Fig. 1. Algorithm for the study of qualitative and quantitative indicators of the organization of collagen fibers in the tissue of benign and malignant neoplasms of the mammary gland

unit area) at which the microscopic examination is performed should be taken into account. Both parameters make it possible to evaluate the functional state of the tissue, namely, its density, strength and elasticity.

Next, we have analyzed such quantitative parameters of collagen fibers as straightness, alignment, and orientation (angle). The straightness of the fibers is presented as a coefficient ranging from 0 to 1, where the size of the value correlates with the degree of straightness of the fibers on the microphotograph. Alignment describes the general tendency of the direction of fibers relative to each other and is also presented as a coefficient ranging from 0 to 1, where the maximum value (1) indicates that all fibers in the tissue are perfectly parallel. The angle value indicates the size of the angle formed by each fiber at the intersection with a conventional line parallel to the horizontal axes of the photograph. In general, the study of the complex of these indicators allows us to identify the peculiarities of the spatial arrangement of collagen fibers, to determine their orientation, regularity and level of anisotropy.

As shown in Fig. 2, collagen fibers in the BCa tissue were more straightened than in FA ($p < 0.001$) — the value of the straightness coefficients were 0.92 and 0.89 respectively. No significant difference was found in the general alignment of collagen fibers in the tissue of benign and malignant neoplasms. At the same time, the angle index in the BCa tissue was significantly higher than that in the FA tissue ($p < 0.05$).

Therefore, collagen fibers in the BCa tissue are characterized by significantly lower values of length ($p < 0.001$) and width ($p < 0.001$), as well as higher values of straightness ($p < 0.001$) and angle ($p < 0.05$) compared to similar indicators in the FA tissue. No significant difference was found in the density of collagen fibers in the tissue of benign and malignant neoplasms of the mammary gland. These results coincide with the data reported by Bodelon *et al*. [24], who analyzed the state of the collagen network in the tissue of benign and malignant breast neoplasms, using the CurveAlign package and the SHG microscopy technique, and established statistically significantly higher indicators of length, straightness, and density in FA tissue compared to the BCa tissue.

To sum up, we have developed and evaluated an algorithm for determining the quantitative characteristics of the collagen matrix of tumor tissue, which can be used as a tool for characterizing changes in the tumor microenvironment during the tumor progression. The advantage of the algorithm is its universality and relative ease of use. The used complex of programs allows the evaluation of a wide range of collagen parameters, including their spatial orientation and mutual arrangement, parametric characteristics and density of the three-dimensional fibrillar network. The algorithm presented in the work and the set of the numerical

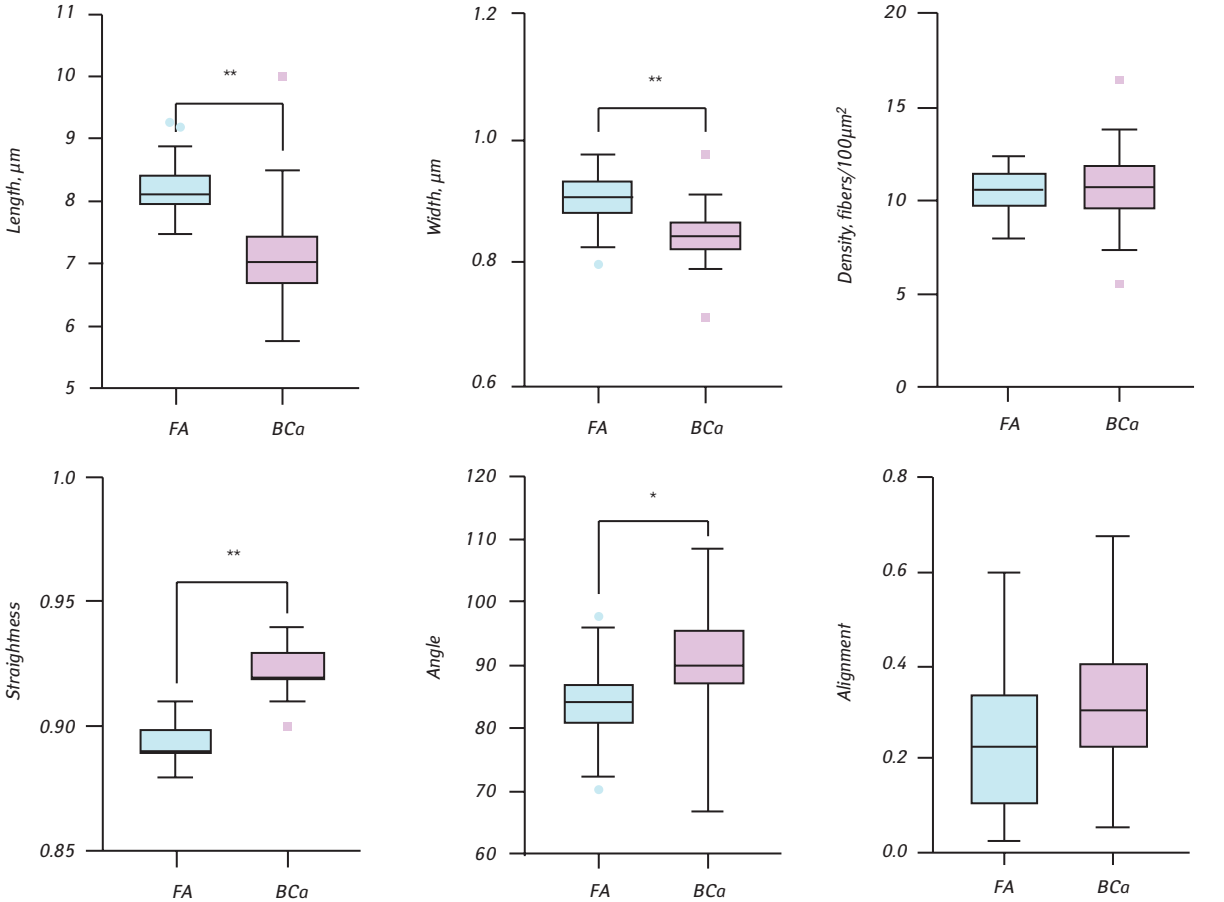

Fig. 2. Quantitative indicators of the spatial organization of collagen in the tissue of benign and malignant neoplasms of the mammary gland: *$p < 0.05$ — the difference is significant in comparison with the corresponding indicator in the FA tissue; **$p < 0.001$ — the difference is significant in comparison with the corresponding indicator of the FA tissue

characteristics and parameters of the topology of extracellular collagen obtained using this algorithm may represent the basis to obtain informational attributes for solving the problems of machine learning and building an intelligent systems with the ultimate goal of improving the differential diagnosis of breast tumors.

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АЛГОРИТМ ДІАГНОСТИКИ ПУХЛИН МОЛОЧНОЇ ЗАЛОЗИ ЗА ПАРАМЕТРАМИ ОРГАНІЗАЦІЇ КОЛАГЕНУ

Н. Лук'янова, Т. Задворний, О. Мушій, Т. П'ятчаніна, В. Чехун

Інститут експериментальної патології, онкології і радіобіології ім. Р.Є. Кавецького НАН України, Київ 03022, Україна

Зміни в кількісних параметрах і просторовій структурі колагену є визнаним і ключовим діагностичним та прогностичним фактором, що асоціюється з розвитком багатьох злоякісних новоутворень, у тому числі і раком молочної залози (РМЗ). ***Метою*** роботи була розробка алгоритму верифікації параметрів дезорганізації колагену як інформативних атрибутів, асоційованих із перебігом РМЗ, з використанням елементів штучного інтелекту. ***Матеріали та методи:*** Дослідження виконано на зразках пухлинної тканини 5 пацієнток з фіброаденомами молочної залози та 20 хворих на РМЗ I–II стадії. Ідентифікацію колагену в тканині новоутворень молочної залози проводили гістохімічним методом за Малорі. Морфометричні дослідження проводили з використанням програмного забезпечення CurveAlign v. 4.0. beta та ImageJ. ***Результати:*** Розроблено та протестовано на зразках операційного матеріалу алгоритм верифікації параметрів дезорганізації колагену як інформативних атрибутів, асоційованих із перебігом РМЗ. Показано, що колагенові волокна у тканині РМЗ характеризуються достовірно нижчими значеннями довжини ($p < 0,001$) та ширини ($p < 0,001$), а також більшою прямолінійністю ($p < 0,001$) та орієнтацією ($p < 0,05$) у порівнянні з аналогічними показниками у тканині фіброаденом. Не виявлено достовірної різниці у щільності колагенових волокон в тканині доброякісних та злоякісних новоутворень молочної залози. ***Висновки:*** Розроблений алгоритм дозволяє оцінювати широкий спектр морфометричних параметрів колагенових волокон в пухлинній тканині, у тому числі визначати їх просторову орієнтацію, а також характеристики та щільність просторової фібрилярної сітки.

Ключові слова: колаген, рак молочної залози, фіброаденома молочної залози.