Evaluation of desmoplasia in pancreatic and colon tumors by morphometric analysis

Avaliação da desmoplasia em tumores de pâncreas e cólon por análise morfométrica

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RESUMO
Objetivos: Este estudo visa quantificar a deposição de colágeno na desmoplasia de tumores pancreáticos e coloniais. Métodos: Amostras de tumores de cólon e pâncreas foram selecionadas do Hospital das Clínicas de Pernambuco e submetidas à tricotomia com Tricrômico de Gomori. A análise fotomicrográfica foi realizada utilizando o sistema integrado de análise de imagens Panthera L. Resultados: Identificou-se que 31 tumores de cólon e 22 tumores pancreáticos apresentaram desmoplasia com a visualização de colágeno fibrilar distribuído irregularmente ao redor do tumor. Houve diferença estatisticamente significativa (p = 0.0031) entre a deposição de fibras colágenas nos tumores pancreáticos desmoplásicos e não desmoplásicos, bem como nos tumores colônicos (p < 0.001). Conclusão: Observou-se que mais da metade dos tumores analisados apresentavam desmoplasia com diferença de densidade de fibrilas de colágeno estatisticamente significativa.

Palavras-Chave: Reação desmoplástica; Câncer; Matriz extracelular; Fibrose; tricrômico de Gomori

ABSTRACT
Objectives: This study aims to quantify collagen deposition in the desmoplasia of pancreatic and colonial tumors. Methods: Samples of colon and pancreas tumors were selected from the Hospital das Clínicas de Pernambuco and submitted to trichomy with Gomori's Trichrome. Photomicrographic analysis was performed using the integrated Panthera imaging analysis system L. Results: It was identified that 31 colon tumors and 22 pancreatic tumors presented desmoplasia with the visualization of fibrillar collagen distributed irregularly around the tumor. There was a statistically significant difference (p = 0.0031) between collagen fiber deposition in desmoplastic and non-desmoplastic pancreatic tumors, as well as for colonic tumors (p < 0.001). Conclusion: It was observed that more than half of the tumors analyzed presented desmoplasia with a difference of density of collagen fibrils statistically significant.

Key words: Desmoplastic reaction; Cancer; Extracellular matrix; Fibrosis; Gomori's trichrome

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INTRODUCTION

The extracellular matrix (ECM) is a dynamic network of proteins capable of regulating the development and maintenance of tissue homeostasis. In addition, it plays a structural role, acts on cellular biochemical signaling, and is able to act as a physical barrier to the process of invasion of tumor cells (RHIM et al., 2014; ZIPPI et al., 2017).

Changes in the ECM modify the tumor microenvironment (TME), mainly due to the installation of a desmoplastic reaction. Desmoplasia comprises the excessive production of stromal cells being composed of cellular and non-cellular components. Cellular components include cancer-associated star cells or myofibroblast-like cells and immune cells. The non-cellular component of tumors consists largely of various ECM proteoglycans and glycosaminoglycan proteins (BRAUCHLE et al., 2018; GARCÍA-PRAVIA et al., 2013; GRADY, MARKOWITZ, 2014).

In the desmoplastic process, the ECM is remodeled mainly by the action of cancer-associated fibroblasts (CAF), which acquire a phenotype capable of positively regulating the interstitial matrix. Thus, there is an increase in deposition and reorganization for crosslinking fibrillar collagen conformation in peritumor regions, especially fibrillar collagen I (BRAUCHLE et al., 2018; RHIM et al., 2014). These changes make the stroma dense and rigid, which triggers mechanotransduction pathways capable of influencing the process of apoptosis, angiogenesis, invasion, migration, and metastasis (BOUGORT et al., 2020; CONKLIN et al., 2011).

In pancreatic tumors, CAFs are identified as quiescent pancreatic star cell activators (PSCs), which contribute to fibrogenesis. This occurs through the activation of several signaling pathways, including sonic hedgehog (HhS), transforming growth factor β (TGF-β), and transformation of growth factor α (TGF-α) (APTE, 2013).

In colon cancer (CC), changes in TME are an important regulatory mechanism for progression and metastasis, since the EMC in the colon devoid of neoplasia is able to restrict the dissemination of metastatic cells (NISSEN, KARSDAL, WILLUMSEN, 2019). Histopathologically, the main characteristics of CC involve invasive growth and changes in the surrounding stroma (KALANTZIS et al., 2020).

The three-dimensional architecture of fibrillar collagen as fibrous capsules, as well as its arrangement of reticulated and elongated fibers, influence tumor plasticity through biochemical and biophysical signaling. Thus, the neoplastic cell activates mechanisms capable of breaking the basement membrane, acquiring an invasive
phenotype guided by the density of fibrillar collagen that controls the direction and speed of migration, with the development of collagen signatures associated with the tumor (BRAUCHLE et al., 2018).

In this sense, matrix tension activates metalloproteases (MMP) - mainly MMP-1, MMP-2, and MMP-9 - to break down fibrillar collagen, in addition to triggering the formation of invasions capable of helping a tumor cell in the process of invasion and metastasis (CONKLIN et al., 2011; ZIPPI et al., 2017). In addition, peritumoral remodeling of the ECM is important in regulating tumor expansion. This is because changes in stromatic collagen promote changes in the density and stiffness of the ECM, which infuse invasion of the neoplastic cell (NEBULONI et al., 2016; ZENGER et al., 2020).

Changes in histopathological examination of tissues have shown an increase in clinical value in relation to diagnoses related to patient survival. In the case, for example, of pancreatic tumors, collagen around malignant ducts aligns differently from collagen around normal ducts and chronic pancreatitis, this characteristic has been associated with TME expression and poor prognosis in pancreatic ductal adenocarcinoma (PDAC) (DRIFKA et al., 2016; EBLE, NILAND, 2014). Furthermore, studies show that in CC the immature desmoplastic response seems to be related to the recurrence of this pathology, as well as to the mortality of patients with stage III CC, but nodal involvement and venous embolism seem to be avoided with the presence of mature stroma (NISSEN, KARSDAL, WILLUMSEN, 2019).

**OBJECTIVE**

The present study aimed to stain and quantify the collagen expressed in desmoplastic tumors of the colon and pancreas.

**METHOD AND MATERIALS**

**Materials**

Ethanol (99% PA, ACS, Modern Chemistry), xylol (PA, ACS, Modern Chemistry), 3-aminopropyltrietoxisilane (99%, SIGMA), acetone (PA, ACS, Modern Chemistry), sliding glass (26x76 mm, thickness 1.0-1.2 mm, Precision Glass), cover glass (24x32 mm, thickness 0.13-10.16 mm, Vision Glass), Gomori Trichromatic Stain Kit - Blue Collagen Stain (38016SS2).
Clinical samples

This is an observational, analytical, and retrospective study. Biopsies of patients diagnosed with pancreatic (n = 22) and colon (n = 31) cancer were selected at the Clinical Hospital from Pernambuco. Biopsy reports were analyzed, with histopathological and epidemiological information.

Histological preparation

Paraffin blocks of primary colon and pancreas tumors were used. 4μm sections were prepared, deparaffinated in two xylene solutions, one at 65°C and the other at room temperature. Then, submitted to hydration in ethyl alcohol (100%, 80%, and 70%). Tissues of whose that presented artifacts, lysis or signs of inadequate processing were excluded.

Tricromome Gomori spot

Gomori's trichromatic staining is used to identify muscle fibers, collagen and nuclei. The solution uses a dye based on a sulfated azo, chromotrope 2R, which has selectivity for the cellular cytoplasm. In addition, it has in its composition selective phosphotungstic acid for collagen, mainly fibrillar collagen. Thus, when phosphotungstic acid binds to collagen, the chromotrope 2R bonding occurs.

This staining technique is characterized by the selectivity of collagen marking in the extracellular matrix. It allows visualization of muscle fibers in red, collagen fibers in blue and cell nucleus in black. The technique allowed visualization of the architecture of the fibrillar components of the tumor ECM. The stained histological sections were analyzed by a qualified examiner and submitted to digital analysis using the Image J software.

Histologically prepared samples were fixed in bouin solution for 30 seconds in a microwave with a power of 300 Watts. They rested for 10 minutes and were rinsed under running water for 5 minutes. The samples were submitted to Bouin solution in a microwave oven at 300 Watts of power for 30 seconds, followed by resting on the solution for 10 minutes, and washed under running water.

Then, the samples were counterstained with Weigert hematoxylin (prepared with equal parts of Weigert hematoxylin A and B at a ratio of 1:1) for 20 seconds in a
microwave oven with 300 Watts of power. They rested for 2 minutes and were rinsed under running water for 2 minutes.

The sections were placed in Gomori’s trichromatic solution for 20 seconds in a microwave with 300 Watts of power and rested for 6 minutes. Sequentially, they were submitted to a solution of acetic acid of 1% for 1 min at 23 ºC. Then the stretches were washed with deionized water and dehydrated in ethanol.

Image analysis

For morphological analysis, the integrated Panthera L image analysis system was used with a monochromatic camera with high Motic sensitivity (MoticEurope, SLU, Barcelona, Spain). This system was available at the Laboratory of Cellular and Molecular Biology of the University of Pernambuco Campus Garanhuns. After staining, each slide was evaluated under an optical light microscope for the spatial arrangement of collagen fibers and color quality. In samples that did not fit the staining pattern, new histological sections were obtained and submitted to a new staining stage with Gomori Trichrome. From each slide, on average, 05 histological fields were selected, whose images were analyzed in a magnification of 100x. The fields were selected from left to right and from bottom to top, obtaining the percentage of collagen by means of automated particle analysis according to the selection and measurement of color-based areas. For morphometric analysis of the images, medium-sized interstitial collagen (in pixels) distributed by field was used, captured on a histological slide using the Image J software with the Threshold Color plug-in, whose values were standardized for all images. Image J allowed the quantification of pixels in the high magnification captures of the slides, after standardization of contrast based on control (histological sections of colon and pancreas devoid of tumor). Such pixels are interpreted as luminous dots that vary in intensity according to the color expression: red, blue, or black. In this way, it is possible to decommage the pixels by the intensity of the color, analyzing each decomposition in a three-dimensional orthogonal plane. To transform pixel clusters of the same intensity into median numerical values, the Threshold Color plug-in was used. Thus, segmentations of the conglomerates of interest were obtained in this study, blue (collagen), which allows predicting the density of collagen present in the McS.
Ethical considerations

This proposal is part of the project "Evaluation of the differential expression of extracellular matrix components as biomarkers of prognosis and diagnosis of neoplasms of the gastrointestinal tract". This project was coordinated by Professor Luiza Rayanna Amorim de Lima and was presented and approved by the Ethics Committee of the University on November 27, 2018. Pernambuco Committee/PROPEGE/UPE, (3,041,764).

Statistical analysis

The results were expressed as ± standard error of the mean. The percentages of tumor desmoplastic capacity were compared by means of the student's t-test. The analyses were performed using GraphPad Prism software version 8.0.2 (San Diego, CA), considering a significance level of 5% for all tests adopted.

RESULTS

The selected samples were submitted to Tricromo Gomori staining (Figure 1). 18 colon tumors and 13 pancreatic tumors presented desmoplasia due to visualization of fibrillar collagen distributed irregularly around the tumor were qualitatively identified. The coloration allowed the visualization of collagen showing blue spots. It was noticed that collagen fibers were distributed irregularly and arranged in large bands, often around the neoplastic area.
Figure 1- Pancreatic adenocarcinoma and moderately differentiated colon adenocarcinoma: A and C represent a photomicrogram of gomori's trichromic staining (100x magnification; 100 μm scale) of pancreatic tumors and colonists, respectively; B and D illustrate the digital morphometric analysis of the pancreatic tumor and colon tumor image, respectively (100x magnification; 100 μm scale).

Source: Coêlho; Ferreira; Silva Filho; Lima (2023)

Analysis of histological data showed that of the 13 pancreatic tumors with desmoplasia, 77% (10) belonged to histological type adenocarcinoma, 8% (1) carcinoma, 8% (1) neuroendocrine and 8% (1) pseudopapillary. Regarding the degree of cell differentiation, it was observed: 31% (4) well-differentiated, 31% (4) moderately differentiated, 31% (4) little differentiated, and 8% (1) not classified. In addition, it was demonstrated that 38% (5) had lymph node involvement. Regarding clinical data, there was a prevalence of 54% of women (7).
Figure 2- Clinical and pathological data of pancreatic tumors

<table>
<thead>
<tr>
<th></th>
<th>Adenocarcinoma (13)</th>
<th>Pseudo papillary (1)</th>
<th>Neuroendócrinos (7)</th>
<th>Carcinoma (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Woman</td>
<td>46% (6)</td>
<td>100% (1)</td>
<td>43% (3)</td>
<td>-</td>
</tr>
<tr>
<td>Men</td>
<td>54% (7)</td>
<td>-</td>
<td>57% (4)</td>
<td>100% (1)</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Head of pancreas</td>
<td>23% (3)</td>
<td>-</td>
<td>14% (1)</td>
<td>-</td>
</tr>
<tr>
<td>Tail of pancreas</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100% (1)</td>
</tr>
<tr>
<td>Unclassified</td>
<td>77% (10)</td>
<td>100% (1)</td>
<td>86% (6)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Degree of differentiation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>31% (4)</td>
<td>-</td>
<td>28% (2)</td>
<td>100% (1)</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>31% (4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Little differentiated</td>
<td>38% (5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Invasive</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Unclassified</td>
<td>-</td>
<td>100% (1)</td>
<td>71% (5)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T1</td>
<td>-</td>
<td>-</td>
<td>14% (1)</td>
<td>-</td>
</tr>
<tr>
<td>T2</td>
<td>23% (3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>T3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100% (1)</td>
</tr>
<tr>
<td>T4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Unclassified</td>
<td>77% (10)</td>
<td>100% (1)</td>
<td>86% (6)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Desmoplasia</strong></td>
<td></td>
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</tbody>
</table>
The analysis of the histological data showed that of the 18 colon tumors with desmoplasia, 44% (8) were moderately differentiated, 50% (9) of the histological type adenocarcinoma, 55% (10) with subserosa invasion, 50% (9) with lymph node involvement and 61% (11) with angiolymphatic invasion.

**Figure 3- Clinical and pathological data of colon tumors**

<table>
<thead>
<tr>
<th></th>
<th>Right Colon (11)</th>
<th>Transverse colon (3)</th>
<th>Left colon (16)</th>
<th>Not classified (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>± 62</td>
<td>±60</td>
<td>±54</td>
<td>54</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Woman</td>
<td>54% (6)</td>
<td>67% (2)</td>
<td>81% (13)</td>
<td>-</td>
</tr>
<tr>
<td>Men</td>
<td>45% (5)</td>
<td>33% (1)</td>
<td>18% (3)</td>
<td>100% (1)</td>
</tr>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>91% (10)</td>
<td>100% (3)</td>
<td>93% (15)</td>
<td>100% (1)</td>
</tr>
<tr>
<td>Adenocarcinoma mucinos</td>
<td>9% (1)</td>
<td>-</td>
<td>6% (1)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Degree of differentiation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>9% (1)</td>
<td>33% (1)</td>
<td>44% (7)</td>
<td>-</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>45% (5)</td>
<td>67% (2)</td>
<td>50% (8)</td>
<td>100% (1)</td>
</tr>
<tr>
<td>Little differentiated</td>
<td>18% (2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>9% (1)</td>
<td>-</td>
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</tr>
</tbody>
</table>
Morphometric study of tumors with desmoplasia revealed a significant increase in the density of collagen fibrils (Figure 4). In the analysis of the expression of desmoplasia in bright spots, it was observed that pancreatic tumors presented clusters of bright blue spots in the order of 29.73 pixels/area. This is related to an estimate of about 2 times the deposition capacity of fibrillar collagen of pancreatic tumors devoid of desmoplasia according to the technique used. Statistical data analysis revealed a significant difference ($p = 0.0031$) in fibrillar collagen density in tumors classified as desmoplastic so compared to non-desmoplastics.
In the morphometric analysis, it was observed that colon tumors with desmoplasia presented quantitative expression of bright spots, in pixels, in the order of 40 pixels/area. This corresponds to a deposition of fibrillar collagen 2 times greater than colon tumors without desmoplasia according to the technique used (Figure 5). Statistical data analysis revealed a significant difference (p < 0.001) in fibrillar collagen density in tumors classified as desmoplastic so compared to non-desmoplastic smoplastics.
Figure 6- Quantification of fibrillar collagen in desmoplastic and non-desmoplastic tumors of colon. * p <0.05.

Comparative analyses of data on fibrillar collagen deposition between desmoplastic pancreas tumors and desmoplastic colon tumors revealed that colon tumors have 1.3 times greater density of fibrillar collagen in their extracellular matrix than tumors of the pancreas.

In addition, no significant association was observed between the deposition of fibrillar collagen in the pancreas and colon tumors with their respective clinicopathological data.

DISCUSSIONS

The epidemiological and histopathological profile of colon tumors revealed a mean age of 56 years, with a predominance of females, involvement of the left colon, and greater distribution in moderately differentiated tumors. Pancreatic tumors were predominantly male and had a well-differentiated histological type of adenocarcinoma. The staining technique used revealed that colon and pancreas tumors have a significant deposition of collagen fibrils.
According to current reports, the risk of developing colon cancer is 4.4% for men and 4.1% for women, with a slight predominance among men. However, in our sample, it was observed that 64.5% (20 patients) are female (JANG, BENINGO, 2019). According to studies, the age group at onset was of individuals over 50 years of age (JANG, BENINGO, 2019; CARAPUÇA \textit{et al.}, 2016; INSTITUTO NACIONAL DE CÂNCER, 2022), which was also evidenced in the present study. In addition, studies indicate a higher incidence of the histological type of adenocarcinoma ( LOBO, GIGLIO, AGUIAR, 2020; JANG, BENINGO, 2019).

Gomori’s trichromatic staining showed that 58% (18) colon tumors had desmoplasia. It was found that collagen fibrils had an altered spatial arrangement: they were arranged in a reticulated manner around the tumors, according to the literature (WANG \textit{et al.}, 2021; JAYASINGH, SIMIANTONAKI, KIRKPATRICK, 2015). In addition, the deposition density of collagen was statistically significant. Thus, it is noted that the expression of fibrillar collagen in colon tumors is associated with a lush desmoplastic reaction. Brauchle \textit{et al.}, (2018) conducted a study demonstrating that, in terms of matrix stiffness, tumor fibers were more rigid than control. In our study, the quantification of tumor fibrillar collagen, in pixels, showed higher fibrillar density, which may be related to the characteristic of greater matrix stiffness.

Studies show that not only the increase in collagen is related to the migration of neoplastic cells. Thus, structural remodeling proved to be able to activate mechanotransduction pathways that contribute to this process. The alteration of fibrillar collagen architecture in the desmoplasic matrix may be related to the regulation of proteins involved in remodeling, such as lysyl oxidase (LOX) and MMP. In addition, changes in amino collagen acids, such as proline to hydroxyproline, have been associated with greater fibril stability, with their consequent structural alignment (JANG, BENINGO, 2019; GRADY, MARKOWITZ, 2014). In our study, we observed that tumors in the desmoplastic colon presented fibril cross-linking, which may be related to structural changes in tumor collagen. Thus, future studies should be conducted to evaluate the impact of the conformal, mechanical and molecular characteristics involved in the desmoplasia process.

As for pancreatic tumors, studies show that the histological type of adenocarcinoma corresponds to 90% of the diagnosed cases, being a rare tumor before 30 years and common in the age group above 60 years. In addition, there is a prevalent effect
in male patients (JANG, BENINGO, 2019; WEINBERG, HANAHAN, 2011). In our study, it was found that, according to the literature, there was a predilection for males and histological type adenocarcinoma.

Pancreatic tumors have a marked desmoplastic characteristic of the stroma. Increased fibrillar density occurs in regions adjacent to the tumor to form fibrous capsules (GIUSSANI et al., 2019; ZIPPI et al., 2017; DRIFKA et al., 2016; BASSAGAÑAS et al., 2014). The photomicrographic study of pancreatic tumors allowed the visualization of fibrillar collagen distributed irregularly and arranged in large bands around the neoplastic area. Thus, the growth of neoplastic cells in pancreatic tumors can be determined by both malignant cells and tumor stroma (BASSAGAÑAS et al., 2014).

Our study demonstrated that pancreatic tumors with desmoplasia presented statistically significant fibrillar collagen deposition when compared to non-desmoplastic tumors. In this sense, the deposition of fibrillar collagen in large peritumor bands may be associated with increased stiffness. This process of active remodeling pathways of biochemical and biophysical signaling allows the tumor cell to trigger invasion mechanisms, such as the opening of pores in the fibrous matrix and the activation of growth factors.

**CONCLUSIONS**

In our study, we were able to identify that more than half of the patients diagnosed with pancreas and colon cancer analyzed presented desmoplasia. In desmoplastic samples, the density of collagen fibrils was significantly higher. These results provide support for future research on the microenvironment of pancreas and colon tumors. This is because the study of physical and biochemical alterations in ECM becomes relevant by the potential for identifying variations that tend to be important in the diagnosis and prognosis of colon and pancreatic cancer. Thus, the discovery of new biological markers, with potential for therapeutic targets, can improve the diagnosis and optimize therapeutic strategies, since there is the premise that the attenuation of desmoplastic reaction and tissue remodeling, in general, can help limit the progression of cancer.

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