

ORIGINAL PAPER



Mast cell density in the primary tumor predicts lymph node metastases in patients with breast cancer

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Abstract

Breast cancer (BrCa) is the most frequent neoplastic disease in female, with high morbidity and mortality. Most of the researches were focused on tumor cells concerning their natural evolution, molecular profile, and potential response to therapy. Few and uncertain data are available about the tumor microenvironment and its impact on the progression of the disease. Mast cells (MCs) associated to BrCa have been reported many years ago, but their real and specific role in the biology of this disease remained elusive. In the current study, we have investigated the predictive role of MCs from the primary tumor on lymph node metastasis on patients stratified based on the molecular classification. We investigated 156 patients with BrCa, stratified as luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) type, basal-like, and unclassified. MCs were identified with anti-MC tryptase antibody in a double immunohistochemical reaction combined with anti-cluster of differentiation 34 (CD34) antibody. Mast cell density (MCD) was calculated based on the hot-spot method, on three fields with maximum density of MCs in each case. The final result was the arithmetic media that was compared with the molecular profile and lymph node metastases. We found no significant correlation between MCD and the molecular profile of the primary tumor, but we noticed a strong correlation between intratumor MCD and lymph node metastases, regardless of the molecular type.

Keywords: breast cancer, immunohistochemistry, lymph node metastasis, mast cell density, molecular classification, prognosis.

Introduction

Breast cancer (BrCa) is the most frequent neoplastic disease in females, and despite significant progress acquired in the field of early diagnosis and therapy, both morbidity and mortality remain high. Although expected, early detection of BrCa is most probably the best method to reduce mortality, as shown by a recent populational study [1]. For many decades, the pathological diagnosis was thought to be the “golden standard” and it had a significant impact in performing a therapeutic strategy. A little bit more than 20 years ago, it was introduced a new classification of BrCa, based on gene analysis and molecular profile [2]. The new molecular classification that includes minimum five different types of BrCa, significantly contributed to our understanding of tumor biology, refined the prognosis, and modified the therapeutic strategy [3].

For more than a century, the main studies on BrCa focused preferentially on malignant cells, and just rarely on the tumor microenvironment. This is somehow surprising, because it was very well known already that the behavior of malignant cells is different, depending on the composition of the medium in which they proliferate [4]. Therefore, the molecular profile governs only in part the natural evolution of tumor cells and their spreading in the lymph nodes and

distant organs. Lymph nodes and distant metastases are very important elements of a bad prognosis and many authors tried to investigate the predictive role of some cells, tissues, or biochemical markers. Over the years, there were noticed many changes in the diagnosis, prognosis, and therapy, including primary tumor resection [5]. Despite there were accumulated a lot of solid and convincing data about the individual behavior of BrCa, there are still controversies regarding the relationship between malignant cells and the microenvironment that should be carefully investigated to clarify some controversial issues.

The tumor microenvironment is a complex structure that includes connective fibers, fix and mobile connective cells, nerve fibers, and blood and lymphatic vessels. Most of them are easily identified in routine histological sections, but besides these elements, there is the ground substance with different characteristics from one case to another and it is a basic component of the tumor microenvironment. In the last years, there were accumulated a lot of data that support the determinant role of the micromedium on the evolution of the tumor, particularly in local progression and in lymph node and distant metastases development [6]. It was noticed that some cells and fibers of the microenvironment react by hyperplasia, and in most of the cases, the substrate of this process is unclear or unknown.

Elastic fibers, macrophages, fibrocytes, tumor infiltrating lymphocytes, and mast cells (MCs) belong to this group of elements. Although in the last two decades there were accumulated some data regarding these cells and fibers, their prognostic value remains uncertain, and consecutively, they did not become therapeutic targets. Moreover, their value to predict lymph node metastases is uncertain or even controversial.

MCs were reported in the tumor microenvironment more than 100 years ago by Ehrlich & Westphal, but their significance in malignant disease in terms of prognosis is still a matter of controversy [7, 8]. The relationships between BrCa and MCs have been investigated by many authors, but the results were significantly different in terms of their number and functional aspects. Some authors reported MCs as elements of good prognosis, and others of bad prognosis. Currently, the MCs reaction associated to BrCa is difficult to explain, despite it is well known a large spectrum of substances stored in the specific granules, which can stimulate or inhibit tumor cell proliferation.

MCs are usual components of the connective tissue and they have been constantly reported in the microenvironment of a broad spectrum of malignant tumors. MCs significantly influence the local composition of ground substance, and by growth factors may stimulate the proliferation of tumor cells. The role of MCs in prognosis of BrCa is still a matter of controversy. In a relatively recent study, in which there were analyzed many publications on this topic, and it was shown that the controversy continues [9]. Only three studies signaled a relationship between MCs density and molecular type of BrCa [10–12]. Experimental studies signaled the involvement of MCs in tumor cells proliferation and spread. So, in an animal model of BrCa, it was found that MCs stimulate tumor growth and limit the basal cytokeratin (CK) 5 compartment [13]. Accumulation of MCs in the presence of annexin 1 was associated with inflammatory response and angiogenesis in triple negative human BrCa [14]. In some human malignant tumors, it was found an accumulation of MCs at the interface with the stroma. It was suggested that MCs form a barrier that restricts invasion based on their content of glycosaminoglycans. On the other hand, MCs as a bad prognosis element seem to be related by their presence in the tumor area, close to tumor cells. This is supported by the deficient response to chemotherapy, particularly in inflammatory carcinoma [15].

Aim

Although many studies investigated the presence and location of MCs in patients with BrCa, mast cell density (MCD) was not explored yet in terms of the relationship with the molecular types and lymph node metastases. In the current study, we investigated the relationship between MCs density, the molecular profile of BrCa, and their predictive role in lymph node metastases. We have shown that MCs density does not correlate with the molecular profile but is a strong predictor of lymph node metastases.

☐ Patients, Materials and Methods

In the present study, there were included 156 patients with BrCa. We used the following criteria for inclusion in the study: details on the staging, classification based on the tumor, node, and metastasis (TNM) system, and the

status of the lymph nodes. The clinical and pathological characteristics of patients are shown in Table 1. There were selected cases diagnosed as ductal invasive carcinoma not otherwise specified, lobular carcinoma, particular forms (like mucinous, medullary, and papillary), with or without local recurrence, with and without axillary lymph node metastases.

Table 1 – The clinical and pathological characteristics of patients (n=156)

Variable	Values	Percent [%]
Age at diagnosis [years]		
• Mean	58.9	
• Range	34–82	
Familial BrCa history		
• Yes	5	3.20
• No	151	96.79
Oral contraceptives		
• Never	62	39.74
• Constant	94	60.25
Menopausal status		
• Premenopause	31	19.87
• Postmenopause	125	80.12
Size of the tumor [cm]		
• Mean	3.8	
• Variation	13.5	
Lymphovascular invasion		
• Present	65	41.66
• Absent	91	58.33
Lymph node status		
• Negative	76	48.71
• 1–3 lymph nodes	44	28.20
• Over three lymph nodes	30	19.23
Grade		
• G1	17	10.89
• G2	81	51.92
• G3	58	37.17
Histopathology		
• Ductal invasive, NOS	130	83.33
• Lobular invasive	6	3.84
• Medullary	7	4.48
• Mucinous	2	1.28
• Metaplastic	9	5.76
• Papillary	2	1.28
Nottingham Prognostic Index (NPI)		
• Less than 3.4	15	9.61
• 3.4–5.4	68	43.58
• Over 5.4	32	20.51
• Not done	41	26.28
Local recurrence		
• Yes	12	7.69
• No	144	92.30

BrCa: Breast cancer; NOS: Not otherwise specified.

Primary processing

Specimens were fixed in 10% neutral buffered formalin for 24–48 hours, pH 7.2–7.4, and embedded in Paraplast High Melt (Leica Biosystems). Step sections, 3–5 µm thick (Shandon, HM355S automatic microtome, Thermo Scientific, USA), stuck on slides were stained with conventional Hematoxylin–Eosin (HE) for the histopathological (HP)

diagnosis and grade. After staining, slides were mounted with Leica CV Mount (Leica Biosystem Newcastle Ltd., Newcastle upon Tyne, UK). The grading was done in accord with *World Health Organization* (WHO) recommendations [16]. Nottingham Prognostic Index (NPI) was calculated based on the accepted formula, considering the size of the tumor, lymph node status, and grade [17].

Immunohistochemistry

All procedures (dewaxing, antigen retrieval, visualization) were performed using Leica Bond-Max (Leica Microsystems

GmbH, Wetzlar, Germany). Briefly, slides were dewaxed in two baths of Bond Dewax Solution five minutes each, followed by rehydration with decreasing alcohols for two minutes each, and distilled water. Endogenous peroxidase was blocked with Dako REAL™ Peroxidase-Blocking Solution for five minutes. Nuclei were stained with modified Mayer's Hematoxylin (HMM500, ScyTek Laboratories, Inc.). Slides were then dehydrated, clarified, and mounted with Leica CV Mount (Leica Biosystems). Details on the primary antibodies, dilution, antigen retrieval, and working system are shown in Table 2.

Table 2 – Antibodies, working system, and expression of the final product

Antibody	Clone	Dilution	Antigen retrieval	Incubation	Working system/ chromogen	Expression
ER	1D5	RTU	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Nuclear
PR	Pgr636	RTU	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Nuclear
Ki67	MIB1	RTU	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Nuclear
HER2	Rabbit polyclonal	RTU	MW, 30 minutes, HercepTest antigen retrieval solution	30 minutes, RT	HercepTest™ visualization reagent, DAB	Membrane pattern
p53	DO7	RTU	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Nuclear
Bcl-2	124	RTU	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Nuclear, cytoplasmic
E-cadherin	NCH 38	1:100	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Membrane/cytoplasmic or both
CK5/6	D5/16 B4	1:80	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Cytoplasmic
CK8	35β H11	RTU	Proteinase K, 5 minutes, RT	30 minutes, RT	LSAB+/HRP, DAB	Cytoplasmic
CK18	DC10	1:25	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Cytoplasmic
EGFR	Polyclonal	RTU	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	EGFR pharmDx™ visualization reagent, DAB	Membrane, cytoplasmic
Mast cell tryptase	AA1	1:300	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, Fast Red	Cytoplasmic, granular

Bcl-2: B-cell leukemia/lymphoma-2; CK: Cytokeratin; DAB: 3,3'-Diaminobenzidine (dihydrochloride); EGFR: Epidermal growth factor receptor; ER: Estrogen receptor; HER2: Human epidermal growth factor receptor 2; HRP: Horseradish peroxidase; LSAB: Labeled Streptavidin–Biotin; MW: Microwave; PR: Progesterone receptor; RT: Room temperature; RTU: Ready-to-use.

Interpretation

Slides stained for nuclear markers, like estrogen receptor (ER), progesterone receptor (PR), and Ki67 were evaluated using the semi-automated method proposed by Suciú *et al.* [18], using the soft NIS-elements D2.30 (Nikon Instruments Europe BV) and the microscope Nikon Eclipse 80i, adjusted with video camera Nikon DS-Fi1 (Nikon Instruments Europe BV). Hormone receptor expression was scored by applying the Allred score [19], which combines the percent of positive nuclei with the intensity of the final product of the reaction. Human epidermal growth factor receptor 2 (HER2) status has been interpreted based on the *American Society of Clinical Oncologists* (ASCO) recommendations, and only +2 and +3 cases were considered positive. E-cadherin-positive reaction was scored according to the system largely accepted in the literature [20]. Only cases scored as +2 and +3 were considered to be positive. Bcl-2 was scored according to the system proposed by Callagy *et al.* [21], and p53 was evaluated based on the recommendations of Yamashita *et al.* [22]. CK5 was performed to characterize basal-like carcinoma, and CK8 and CK18 were done to identify micrometastases in the lymph nodes. Epidermal growth factor receptor (EGFR) was evaluated based on the recommendation of Dako guide (EGFR pharmDX™, Dako, Denmark). The reaction was considered positive if more than 5% of tumor cells were positive.

Mast cell density

MCD was evaluated using a system similar to that

proposed by Weidner to count microvessels. There were chosen three microscopic fields with a maximum density of MCs in the tumor area, at a magnification of ×400. There were counted MCs stained for MC tryptase and the arithmetic media was the final result for each case. Overestimation of MCD values is less probable, based on the high specificity of the antibody used in the current study. Values of MCD were correlated then with the HP form, grade, molecular types of carcinoma and lymph node metastases.

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 22.0 software (SPSS Inc., Chicago, IL, USA). Student's *t*-test was applied and a value of $p < 0.05$ was considered significant.

Results

MCs were found in all the cases included in the present study, but their density was significantly different from one case to another. MCs were noticed in both intratumor and peritumor areas but associated with different components of the tumor. In the peritumor area, MCs were frequently observed in the perivascular space with limited variation in number from one field to another (Figure 1A). MCs in the tumor area were often found in close contact with tumor cells and just rarely located in the perivascular space. In some cases, few MCs were found in the tumor area (Figure 1B), but in most of the cases, their number was

higher than in the peritumor area. On occasion, we found numerous MCs agglomerated as a continuous line at the interface between neoplastic cells and the connective tissue of the microenvironment (Figure 1C). The release of specific granules of MCs in the pericellular space, known as degranulation, was noticed in many cases (Figure 1D), particularly in the peritumoral space and never in the intratumor area. No significant correlation was found between degranulation and lymph node metastases ($p < 0.22$) or the molecular profile of the primary tumor ($p < 0.71$). The highest

value for MCD in the intratumor area was found in cases in which tumor cells were arranged in small groups, usually discohesive (Figure 1, E and F). We found no relationship between the number of intratumor and peritumor MCs and the conventional HP type of carcinoma. Although MCs were most numerous in ductal invasive carcinoma, and just rare in mucinous carcinoma, they were not good indicators of the pathological form. We found a slight correlation with the grade, with values of MCD increasing as G increased ($p < 0.047$).

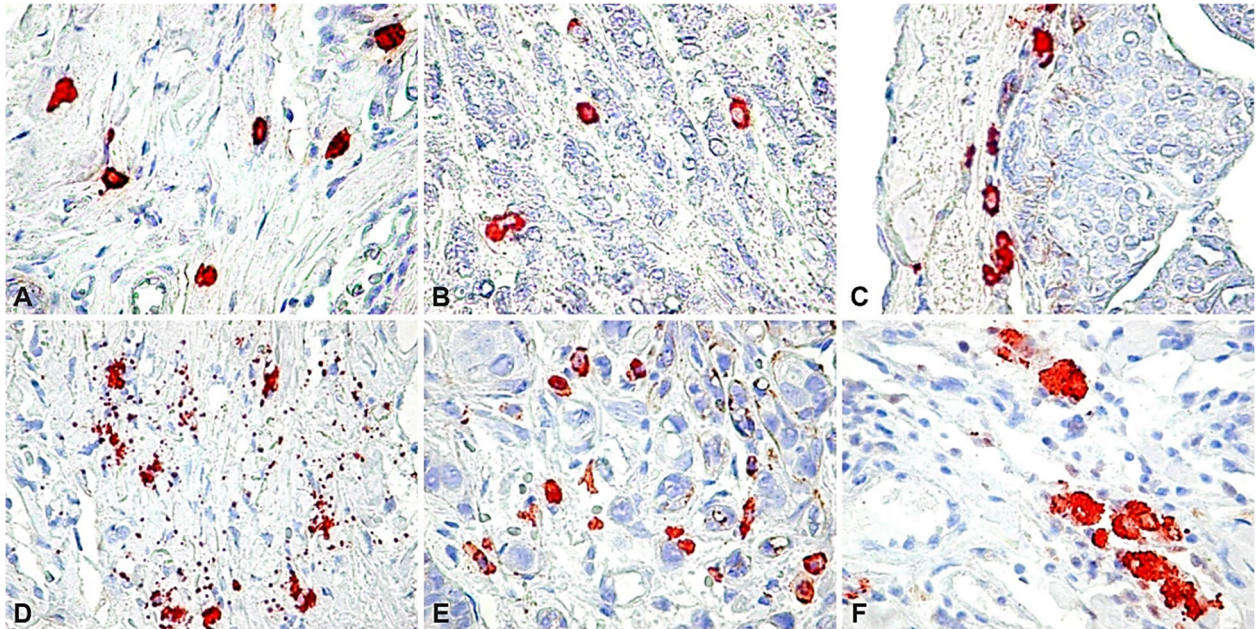


Figure 1 – (A) MCD in the peritumor microenvironment; (B) Low MCD in the tumor area; (C) Borderline MC barrier; (D) Release of MC granules in the peritumor area; (E) High MCD in the tumor area; (F) Details, MCs agglomeration. MC tryptase immunoreaction: (A–E) $\times 400$; (F) $\times 900$. MC: Mast cell; MCD: Mast cell density.

We then analyzed the relationship between MCD and the molecular types of BrCa. Luminal A was found in 73 of the cases, based on the strong expression of ER and PR, Bcl-2, low Ki67 expression, and negative reaction for HER2. Luminal B cases ($n=26$) showed expression for hormone receptors, usually lower than in luminal A, with a high Ki67 index and negative reaction for HER2 protein. Basal-like carcinoma (16 cases) was characterized by the expression of CK5, EGFR and p53, and negative reaction for hormone receptors and HER2. Finally, unclassified cases ($n=8$) were negative for all markers included in this study. We found the highest values for MCD in luminal A and HER2 types of BrCa, but without a correlation with the global prognostic impact of the molecular type (Table 3). The lowest value for MCD was found in the basal-like carcinoma, well-known for the reduced rate of metastases in the axillary lymph nodes.

Table 3 – Correlation between MCD and molecular types of breast carcinoma

Molecular type ($n=156$)	MCD/mm ²		p-value
	Intratumor MCD	Peritumor MCD	
Luminal A ($n=73$)	120.09	83.16	0.025
Luminal B ($n=28$)	111.84	73.17	0.797
HER2 ($n=31$)	129.69	71.94	0.904
Basal-like ($n=16$)	57.84	105.33	0.029
Unclassified ($n=8$)	74.13	74.55	0.987

HER2: Human epidermal growth factor receptor 2; MCD: Mast cell density.

An aspect with significant clinical importance resulted from the analysis of the relationship between MCD and axillary lymph node metastases. We found a strongly statistically significant relation between the high number of MCs in the tumor area only and lymph node metastases (Table 4). No correlation was noticed between the peritumor MCD and lymph node metastases.

Table 4 – Correlation between MCD and axillary lymph node status

Lymph node status	MCD in the primary/HPF	p-value
Positive lymph nodes	56.47	<0.0001
Negative lymph nodes	16.22	<0.0001

HPF: High-power field ($\times 400$); MCD: Mast cell density.

Discussions

In the current work, we have shown that MCs are important players in the behavior of BrCa. Their heterogeneous distribution and morphology most probably reflect the ability of these cells to secrete a broad spectrum of biologically active substances. Therefore, MCs should be considered an essential component of the tumor microenvironment. The tumor microenvironment is a complex structure that includes fixed and wandering connective tissue cells, fibers, blood, and lymphatic vessels. In the last years, there were accumulated many data that support the determinant role of the microenvironment in the tumor

evolution, contributing to both local progression, lymph node, and distant metastases [23]. We have investigated the MCs because of their high capacity to react to pathological conditions.

MCs are usual components of the loose connective tissue, and they were constantly reported in the tumor microenvironment. It is difficult to discriminate between “good MCs” and “bad MCs”, because the active biological substances contained in the specific granules are different. Therefore, MCs form a heterogeneous cell population that influences not only the vascular dynamic and composition of the ground substance, but also stimulates the proliferation of tumor cells by secreting growth factors.

The prognostic role of MCs in BrCa is very controversial. So, in a relatively recent study on this topic, analyzing many publications, it was concluded that in some conditions MCs are of good prognosis, and in others, of bad prognosis [9, 24]. Few studies investigated the relationship between the MCD and molecular type of BrCa. We found no relationship between the molecular type and MCD. Therefore, we believe it is important to identify a particular signature of BrCa. On the other hand, some studies signal the involvement of MCs in tumor cell proliferation and spreading. In an animal model of BrCa, it was noticed that MCs stimulate tumor growth and limit the development of the basal CK5 compartment [25].

These data are supported by our findings, which signal the predictive role of MCs number in the primary tumor to lymph node metastases. In their turn, these data are supported on triple-negative tumors, in which MCs infiltrates in the presence of annexin 1 is associated with inflammation and angiogenesis [26]. In some human malignant tumors, it was reported the agglomeration of MCs at the front of tumor proliferation. It was thought that MCs have the tendency to form a barrier against tumor invasion, particularly using secreted glycosaminoglycans. Our results do not confirm this hypothesis, although we found some aspects of this kind. The location of MCs in the immediate vicinity of tumor cells is most likely a local adaptation to the release of some growth factors, like vascular endothelial growth factor (VEGF). It is well-known that in an advanced stage, BrCa tumor cells do not express VEGF, and we believe that this growth factor is secreted by cells of the microenvironment, like MCs.

A bad prognosis is suggested by the accumulation of MCs in the intratumor area is given by the lack of response to chemotherapy, particularly in inflammatory BrCa [15, 27]. The unfavorable prognosis of tumors with MCs infiltrates has been demonstrated in a large variety of human cancers [28, 29]. Based on these data, we believe that MCD is a useful method to predict lymph node metastases and bad prognosis in patients with BrCa. In these conditions, MCs become interesting candidates for target and personalized therapy.

MCs and conventional clinicopathological data

We did not find a correlation between MCD and the HP form, and just a weak correlation with the grade of the tumor. Our data confirm previous findings related to this topic that show the increased number of MCs in high-grade BrCa [30]. This could be indirect support for the hypothesis that a high number of MCs in the intratumor area is of bad prognosis.

MCs and molecular classification

Very few data are available about the relationship between MCs and the molecular type of BrCa. In the current study, we found no correlation between these parameters, and we cannot confirm previous data published on this topic [12, 31–33]. This could be due to the limited number of patients included in these studies and the use of a restricted panel of antibodies to characterize the molecular types of BrCa.

MCs and tumor cells

The role of MCs in the tumor microenvironment is still elusive. The association between MCs and tumor cells has been reported by many authors [34–36]. The close apposition of MCs to tumor cells could be the morphological expression of a very important step in the evolution of neoplasia: the release of growth factors that MCs is able to secrete. This could be an explanation for the location of MCs in the tumor area in the proximity of tumor cells but not around blood vessels. This is supported by the lack of degranulation in the tumor area because growth factors, like VEGF, are released usually by diffusion. MCs are known for their pivotal role in tumor-associated angiogenesis, as they secrete several angiogenic factors, we hypothesize that MCs secrete an important amount of VEGF and contribute to the formation of new blood vessels. This is also supported by the fact that BrCa tumor cells do not secrete VEGF in an advanced stage, as it was the case in the current study. Taken together, these data suggest that intratumor MCs are of bad prognosis, as also has been shown by other publications [8, 37]. In this condition, MCs inhibitors could be of interest in the clinical practice of patients with BrCa [38].

To the best of our knowledge, it is the first demonstration of the predictive role of MCs from the intratumor area on axillary lymph node metastases. MCs could be the source of growth factors that can induce lymphangiogenesis in the tumor area and thus facilitating the spread of malignant cells on the lymphatic route. In such a condition, MCs became an attractive therapeutic target, and inhibitors of MCs degranulation could be considered as adjuvant therapy in BrCa.

Conclusions

In the present study, we have investigated the distribution location and number of MCs in 156 specimens of BrCa. MCD from the tumor and the peritumoral area does not correlate with the conventional HP diagnosis, the molecular profile of BrCa included in the current study and shows just a slight correlation with the grade of the primary tumor. On the other hand, the MCD in the tumor, but not in the peritumoral area, statistically and significantly correlated with lymph node metastases, regardless of the molecular type of the tumor. Based on our data, the MCD from the tumor area is a good predictor for lymph node metastases.

Conflict of interests

The authors declare that they have no conflict of interests.

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Authors' contribution

EF, design of the study, analysis, and specimen collection; ICNT, specimen collection; ARC, RMC, performed immunohistochemistry and quality assurance; RAP, writing the manuscript; NPG, MR, supervisor, and writing the manuscript.

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