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Morphological and Immunophenotipical Characterization of Murine Mammary Carcinoma 4t1

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Abstract

The 4T1 murine mammary carcinoma is an experimental model widely used in assessing and better understanding of tumor biology. It is a highly tumorigenic cell line and invasive, where metastases are observed in various organs. This study aims to describe morphological and immunophenotipical aspects of 4T1 mammary carcinoma in mice Balb/c with the aid of the immunohistochemistry. Tissues were fixed in formalin and processed using the routine paraffin inclusion technique. Histologic sections (4 µm) were stained through Hematoxylin-Eosin techniques for morphologic assessments. For immunohistochemical study, we used a panel of 9 (nine) antibodies: hormone receptors, receptors for cell proliferation, cytokeratins, vimentin, growth factor receptor and markers of blood vessels. Morphologically, the 4T1 murine mammary carcinoma shows malignant epithelial proliferation in solid arrangement, with pleomorphic cells and high mitotic index. In immunohistochemical analysis was determined a positivity for hormone receptors, cytokeratin AE1/AE3, receptors of cell proliferation and markers of blood vessels. There was a negative for vimentin, cytokeratin 5/6, cytokeratin 34βE12 and growth factor receptor.The results show that the characteristics observed in this model are similar to some types of breast cancers found in women like poorly differentiated invasive ductal carcinoma. Thus, the immunophenotypic characterization of mammary carcinoma 4T1 allows a better understanding of the model to the study of new anticancer therapies.

Key words: mouse, mammary gland, 4T1 cells and immunohistochemistry.

Introduction

The experimental transplantable tumors have been used in different studies of carcinogenesis (11,27). These tumors are considered appropriate models, and valid in the study of the progression and cancer therapy in different studies (31).

In addition, animals used as a study model have similarities in their physiology, biochemistry, metabolism and genetics in relation to humans, which ensures their use in research contributing to the discovery of new treatments.

Among the various experimental tumors used in research, we highlight the 4T1 murine mammary carcinoma. 4T1 is a highly tumorigenic cell line, little immunogenic with characteristics of growth and metastasis of tumor cells similar to human mammary gland in stage IV (31). Morphologically, the 4T1 mammary carcinoma shows malignant epithelial growth in solid arrangement, characterized by the proliferation of pleomorphic cells and high mitotic index.

Previous studies with the 4T1 tumor demonstrate possibilities for its use as a model in experimental cancer therapy (20, 37). However, research is needed to establish

appropriate proposals for oncological evaluation of this model.

In humans some immunohistochemical markers are known to assist in the characterization of breast cancer, contributing to the predictive and prognostic evaluation of these tumors. These markers include estrogen receptor (ER) (7, 23), progesterone receptor (PR) (7, 23), cytokeratin 5/6 (41), cytokeratin 34βE12 (15), HER-2 (28), MIB-I (38), CD31 (8).

At present, immunohistochemistry is accepted as standard evaluation method worldwide. The immunohistochemistry detects protein products in tissue sections. It enables us to observe protein expression at the individual cell level in the tumor mass (40). With the advent of this tool, the immunophenotypic profile may be useful in understanding the biological character of tumors. In this sense, to our knowledge, this is the first work that describes the morphological and immunophenotypic characteristics of the 4T1 murine mammary carcinoma.

Therefore, this paper aims to describe the immunophenotypic profile of 4T1 murine mammary carcinoma by immunohistochemical technique.

Materials and Methods

4T1 cells (American Type Culture Collection, Manasssas, VA) were maintained in RPMI 1640 medium (Hyclone, Logan, UT) containing 10% FBS, at 37oC in a humidified atmosphere of 5% CO2.

Five female Balb/c mice (6 Weeks, 20g weight) were provided by the Animal Facility Center – Institute of Biological Science – Federal University of Minas Gerais; Belo Horizonte -Brazil. Housing, anesthesia and care were provided according to guidelines established by local Institutional Animal Welfare Committee.

4T1 cells in log phase of growth were harvested and suspended in PBS at a density of 2.5 x 106 cells/mL. 100 µl of the suspension was injected in the right posterior flank of all animals, to obtain a solid tumor.

After 28 days, necropsy was performed with removal of tumor for histopathological analysis. Tissues were fixed in formalin (10% w/v in phosphate-buffered saline – PBS pH 7.4), processed using the routine paraffin inclusion technique (30). Histologic sections (4 µm) were stained through Hematoxylin-Eosin techniques for morphologic assessments.

Furthermore, sections (4 µm) were obtained and mounted on silanized slides for immunohistochemical study. A panel of antibodies was used (Table 1). Heat-induced epitope retrieval using Dako antigen retrieval solution, pH 6.0 (Dako, Carpenteria, CA, USA), was performed before dipping the sections in a water bath (20 minutes). The slides were then cooled at room temperature for 20 minutes in the antigen retrieval buffer. The sections were incubated at room temperature in 3% (vol/vol) H2O2 for 15 min, in primary antibodies for 16 h at 4ºC or for 1h at room temperature (Table 1), in a reagent containing secondary antibodies (Advance HRP link, Dako, Carpenteria, CA, USA) for 30 min and a polymer reagent (Advance HRP enzyme, Dako, Carpenteria, CA, USA) for 30 minutes. Between incubations, the slides were washed for 2 × 5 minutes in 10% phosphate-buffered saline solution. The immunoreactivity was visualised using diaminobenzidine (DAB substrate chromogen system, Dako, Carpenteria, CA, USA). The slides were then counterstained with Harris haematoxylin.

Table 1. Panel of antibodies used for immunohistochemistry analysis

Target antigen	Clone	Dilution	Manufacturer	Incubation (Hr) Primary antibody
Cytokeratin AE1/AE3	AE1AE3	1:30	Dako (Carpenteria, CA, USA)	1
Cytokeratin 5/6	D5/16B4	1:50	Dako (Carpenteria, CA, USA)	16
Cytokeratin 34βE12	34β12	1:40	Dako(Carpenteria, CA, USA)	1
Vimentin	V9	1:50	Dako(Carpenteria, CA, USA)	1
ER	1D5	1:30	Dako (Carpenteria, CA, USA)	16
PR	hPRa2	1:20	Neomarkers (Fremont, CA, USA)	1
CDC 47	47DC141	1:300	Neomarkers (Fremont, CA, USA)	1
HER-2 (polyclonal)	NA	1:200	Dako (Carpenteria, CA, USA)	16
CD31	HC1/6	1:40	Fitzerald (Acton, MA, USA)	1

NA: Not Applied

For determining of the expression of ER and PR was used American Society of Clinical Oncology / College of American Pathology (ASCO / CAP) (16), the evaluation of CDC47 was used orientation Souza et al. (36) and CD31 was evaluated according Maeda et al. (24). Evaluation of cytokeratin 34βE12 was performed according to Ferreira et al. (10). Vimentin and cytokeratin AE1/AE3 antibody was used orientation adapted of Bettini et al. (5). For determining the expression of HER-2 was used the American Society of Clinical Oncology / College of American Pathology (ASCO / CAP) (42).

Positive and negative control slides were included in each batch. As a positive control we used mouse mammary gland known to express of antibody (except HER-2). As positive control for this antibody was used a human mammary carcinoma with overexpression previously known for HER-2. Negative controls were assessed using normal serum (Ultra V Block, Laboratory Vision) as the primary antibody.

Table 2. Immunohistochemical expression of ER, PR, HER-2, CK34βE12, CKAE1AE3, CK5/6 and vimentin in murine mammary carcinoma 4T1

Antibodies	0	+	++	+++
ER[a]	0/5	5/5	NA	NA
PR[a]	0/5	5/5	NA	NA
HER-2[b]	5/5	0/5	0/5	0/5
CK34βE12[c]	5/5	0/5	NA	NA
CKAE1AE3[c]	0/5	5/5	NA	NA
CK5/6[c]	5/5	0/5	NA	NA
Vimentin[d]	5/5	0/5	NA	NA

ER, Estrogen Receptor; PR, Progesterone Receptor; CK, Cytokeratin; NA, Not Applied.
[a] 0 < 1% nuclear staining of tumor cells; + > 1% nuclear staining of tumor cells.
[b] 0 No staining; + weak, incomplete membrane staining of any proportion of the tumor cells; ++ complete membrane staining that is either non-uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells, or intense, complete membrane staining of 30% of tumor cells.
[c] 0 No staining; + cytoplasmic staining in epithelial cells.
[d] 0 No staining; + cytoplasmic staining in mesenquimal cells.

All procedures were performed under the guidelines and with the approval of the Ethics Committee in Animal Experimentation (CETEA/UFMG), protocol 201/09.

Results

All tissue sections were composed of proliferation of the epithelial cells organized in a solid arrangement, with the formation of cords, sheets or clusters surrounded by a fine fibrilar collagenous stroma (Figure 1A). The tumor cells exhibit moderate pleomorfism and have large, round or ovoid, often vesicular nuclei with single or multiple prominent nucleolus, abundant cytoplasm eosinophilic and indistinct cell borders. The mitotic rate ranged from two to three mitoses per 400x field and atypical mitoses were found. Tumors smaller than 0.5cm were central necrotic areas and inflammatory infiltrated composed of neutrophils and lymphocytes.

The immunohistochemical results for ER, PR, HER-2, CK34βE12, CKAE1/AE3, CK5/6 and vimentin staining are shown in Table 2. All of the cases evaluated showed cytoplasmic staining for CKAE1/AE3 (Figure 1B), but were stained negative for cytokeratin 5/6 (5/5), cytokeratin 34βE12 (5/5) and vimentin (5/5). The tumors cells showed nuclear expression of hormonal receptors, ER (5/5) (Figure 1C) and PR (5/5). Negative staining for HER-2 was observed in all of the cases. The proliferation index determined from the amount of nuclear staining for CDC47 ranged from 33.8% to 61.2% (mean 47.8%) (Figure 1D). The assessment of the vascularization of the tumors performed by determining the number of vessels in sections (five animals) staining for CD31 was of 13.96 vessels/field.

Discussion

Numerous models have been developed for breast cancer research. All of these models to address key elements in the biology of breast cancer development and progression (18). The 4T1 murine mammary carcinoma is a widely used experimental model for characterization of cellular and molecular events during neoplasic progression (20,37). However, in literature there are few data on the morphological and immunophenotypical characteristics of 4T1 murine mammary carcinoma.

Morphologically, the 4T1 mammary carcinoma shows malignant epithelial growth in solid arrangement, characterized by the proliferation of pleomorphic cells and high mitotic index. Our results show that the characteristics observed in this model are similar to some types of breast cancers found in women like poorly differentiated invasive ductal carcinoma (32).

Some factors determine the histogenesis and the biologic behavior of neoplasms as hormone receptors, receptors for cell proliferation, cytokeratins, vimentin, growth factor receptor and markers of blood vessels (7,8,28,38). In this study, we evaluated the immunohistochemical expression of several marker proteins.

Staining positive for cytokeratin AE1 / AE3 confirm the epithelial origin of neoplasia. Cytokeratin 5/6 and cytokeratin 34βE12 stain the basal layer of normal breast ductal epithelium (15,41) . CK 34βE12 is a widely used robust monoclonal antibody that identifies a number of high-molecular-weight keratins. CK 34βE12 recognizes basal keratins, including CK1, CK5, CK10 and CK14. CK5 and CK14 are widely used markers of basal

phenotype (25). In present study, the immunostaining for cytokeratin 5/6 and cytokeratin 34βE12 was negative.

Metha et al. (25) showed that expression of 34βE12 predicts disease-free and overall survival in patients with triple-negative tumors (negative immunostaining for estrogen, progesterone and HER-2 receptors). Furthermore, it was observed that women with triple negative tumors that expressed cytokeratin 34βE12 were associated with good prognosis.

Estrogen and progesterone have a role in normal mammary gland development and these hormones are implicated in the development of mammary tumors (23). In present study the tumors cells showed expression of hormonal receptors, ER and PR. Pervez et al. (29) described that expression these receptors relate to the degree of differentiation, the presence of metastases and response to hormonal therapy. Women with positive estrogen and receptor progesterone breast cancer has better prognosis than women with breast cancer negative estrogen and progesterone (3).

Immunostaining of MIB-1 is widely used to assess tumor proliferation, and is an established prognostic factor in breast cancer. However, the evaluation of cell proliferation in an animal model most frequently used protein CDC47. Immunohistochemical expression of intranuclear CDC47 protein has been widely studied, and is associated with the stimulation of gene duplication necessary for cell division (9,26). The expression of this receptor is related with cells that are cycling (predominantly G1 and S phases) (19). Our results demonstrated a high proliferation index. In human medicine, Spyratos et al. (38) described that breast cancers expressing high levels of MIB-1 are associated with worse outcome.

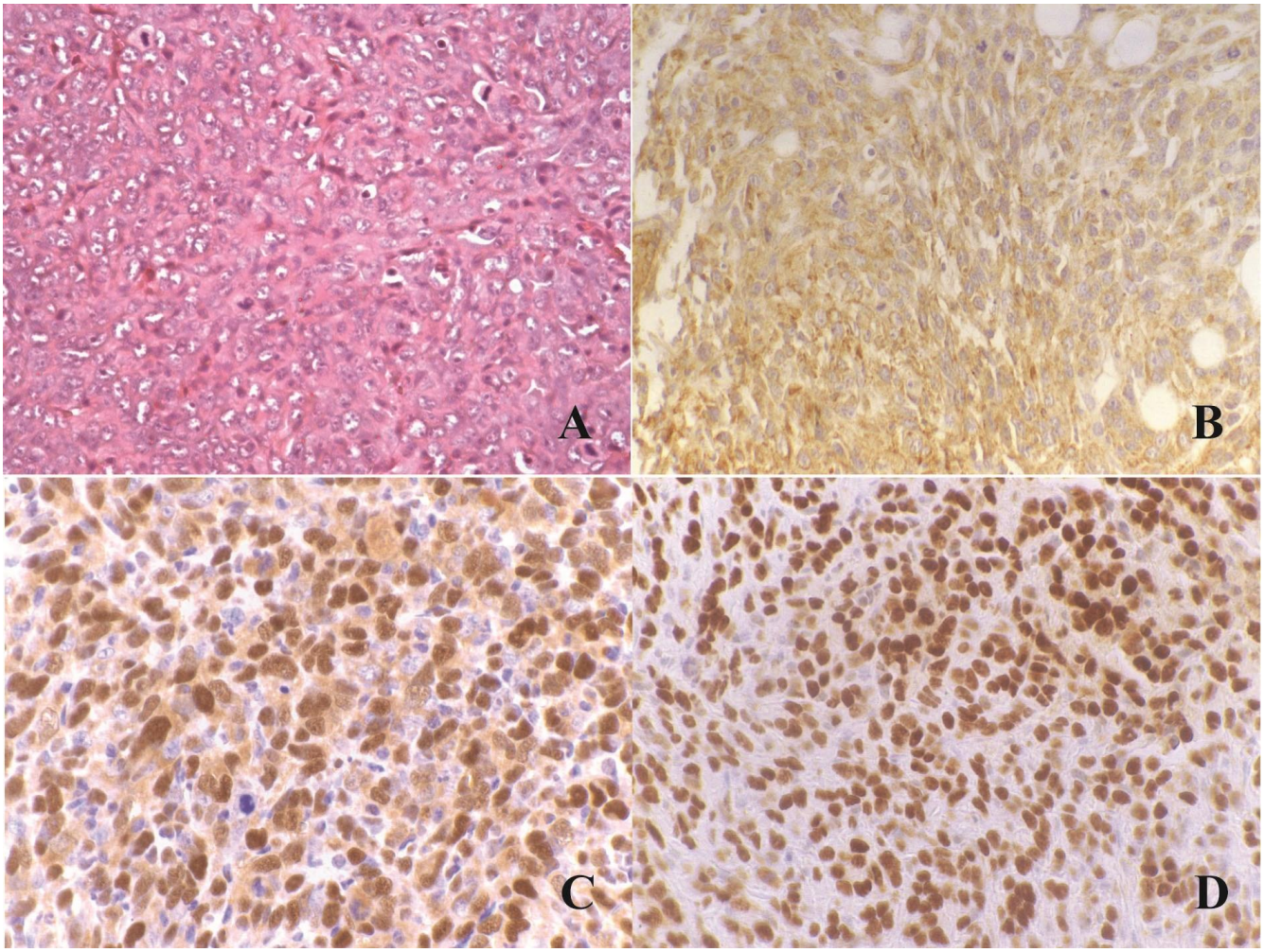

Figure 1. 4T1 tumor. (A) Histopathological analysis tumor 4T1. Observed proliferation of the epithelial cells organized in a solid arrangement. HE, Obj. 40x. **(B)** Tumor cells characterized by membrane immunostaining for cytokeratin AE1/AE3. Chromogen: DAB. Mayer's Haematoxylin counter stain, Obj. 60x. **(C)** Tumor cells characterized by nuclear immunostaining for ER. Chromogen: DAB. Mayer's Haematoxylin counter stain, Obj. 60x. **(D)** Tumor cells characterized by nuclear immunostaining for CDC47. Chromogen: DAB. Mayer's Haematoxylin counter stain, Obj. 60x.

Another important marker CD31 was assessed. The CD31 antibody widely used in the evaluation of vascularization in both normal and neoplastic tissues. Reaction with protein (antigen) localized in the cytoplasm, have clinical applicability as a prognostic factor in several carcinomas, as well as being appropriate in the analysis of angiogenesis in breast tumors (12), colorectal cancer (39) and lung cancer (13). In murine mammary carcinoma 4T1 was observed expression significant of vessels. These results are consistent with human medicine that correlates more angiogenic tumors are more aggressive and worse prognosis (8,12).

Changes in the structure and expression of proto-oncogenes are involved in the origin and progression of tumors. One mechanism is the amplification and overexpression of members of the protein kinase gene family (33). Several tyrosine kinase genes, including HER-2 are amplified in human breast cancer (34). In this study, the immunostaining for HER-2 was negative. These findings are similar to results reported by Heisig et al. (17). These authors used the 4T1 cell line as a negative control for models of stable transfection of her-2 gene. The degree of HER-2 amplification appears to have an effect on survival, with greater copy number being associated with a worse prognosis (34).

Therefore, the results suggest that the immunohistochemical murine mammary carcinoma 4T1 resembles a luminal subtype, as described in humans (6). These authors used immunohistochemistry following definition: basal-like (ER−, PR−, HER-2−, cytokeratin 5/6+, and/or HER-1+), HER-2+/ER− subtype (HER-2+, ER−, PR−), luminal A (ER+and/or PR+, HER-2−), and luminal B (ER+ and/or PR+, HER-2+). These human breast cancer subtypes predict distinct clinical outcomes (35). Thus, the results of this study may also corroborate studies using the 4T1 tumor as a comparative model for the investigation of treatment of breast cancer receptor-positive ER (14, 43).

Curiously, these results were different of the immunohistochemical profile of the primary tumor and metastasis 4T1 as described by Bao et al. (4) and Kau et al. (21), respectively. These authors demonstrated that the metastasis and 4T1 cell line was negative for ER and PR and did not overexpress HER-2 (triple-negative). A possible explanation for this discrepant result may be the fact that they performed their assessment in 4T1 cell lines culture. Furthermore, the assessment of markers used was based on flow cytometry and Western blot analysis. Some authors describe that hormone receptor (ER and PR) status and HER-2 score are differently expressed between the primary tumor and corresponding metastases (1,2), therefore it is reasonable to assume that in vivo and in vitro tumors may present different protein expressions.

Another interesting fact is related with to similarity of immunophenotypic profile of 4T1 tumor with lobular carcinoma. Lakhani et al. (22) describe that the lobular carcinoma are frequently positive for ER and PR and rarely overexpress HER-2. Furthermore, in contrast to the majority of ductal lesions, lobular neoplasia is negative for E-cadherin in about 80-90% of cases. Approximately 10-16% of cases will express E-cadherin, although this is usually aberrant and this pattern should not be used to make a diagnosis of ductal carcinoma.

The results show that the characteristics observed in this model are similar to some types of breast cancers found in women like poorly differentiated invasive ductal carcinoma. Thus, the immunophenotypic characterization of mammary carcinoma 4T1 allows a better understanding of the model to the study of new anticancer therapies.

Conclusion

Based on the presented data, we may conclude that the morphological analysis of the 4T1 murine mammary carcinoma demonstrated characteristics of a poorly differentiated carcinoma. Similar characteristics can be observed in many cases of women breast cancer. Thus, morphological and immunophenotypical characterization of the 4T1 mammary carcinoma promotes a better understanding of the murine model for women breast cancer and enables tumoral behavior and new anticancer therapy studies.

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