Immunohistochemical features of claudin-low intrinsic subtype in metaplastic breast carcinomas

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A B S T R A C T
Purpose: The claudin-low molecular subtype of breast cancer includes triple negative invasive carcinomas, with a high frequency of metaplastic and medullary features. The aim of this study was to evaluate the immunohistochemistry expression of claudins in a series of metaplastic breast carcinomas. We also assessed other claudin-low features, such as the cancer stem cell-like and epithelial-to-mesenchymal transition phenotypes.

Results: The majority of the cases showed weak or negative staining for membrane claudins expression. We found 76.9% (10/13) low expressing cases for claudin-1, 84.6% (11/13) for claudin-3 and claudin-4, and 92.3% (12/13) for claudin-7. Regarding the cancer stem cell marker ALDH1, 30.8% (4/13) showed positive staining. We also showed that the majority of the cases presented a CD44+/CD24−/low phenotype, positivity for vimentin and lack of E-cadherin expression. Interestingly, these claudin-low molecular features were specific of the mesenchymal component of metaplastic breast carcinomas, since its frequency was very low in other breast cancer molecular subtypes, as luminal, HER2-overexpressing and non-metaplastic triple negative tumors.

Conclusions: The negative/low expression of claudins and E-cadherin, high levels of vimentin, and the breast cancer stem cell phenotype suggests that metaplastic breast carcinomas have similar features to the ones included in the claudin-low molecular subtype, specially their mesenchymal components.

Introduction

The claudin-low intrinsic subtype of breast cancer is a group of tumors recently identified by molecular gene profiling. These tumors were characterized by low expression levels of cell-cell junction proteins, such as claudins-3, -4, -7, occludin and E-cadherin. In addition, tumors were significantly enriched in epithelial-to-mesenchymal transition (EMT) features, presenting also high expression of stromal-specific and lymphocyte- or granulocyte-specific gene signatures. Interestingly, it has also been reported that cancer stem cell (CSC)-like characteristics are highly found within this claudin-low molecular subtype, meaning that these tumors proliferate and propagate based in a large population of tumor cells with stem-like cells.

Claudins (CLDNs) are membrane proteins that have key roles in the structure and function of tight junctions (TJs), especially in the maintenance of cell polarity and regulation of paracellular permeability. The CLDN family comprises 27 members, ranging in size from 22 to 27 kD, and topologically categorized by four transmembrane protein classes with the carboxyl-terminus in the cytoplasm and two extracellular loops. Most tissues express multiple CLDNs that can interact in either a homotypic or heterotypic fashion to form the tight junction strand. Although the full CLDNs expression profile in tissues is still not well characterized, it is clear that changes in their expression occur in various tumors.

Interestingly, around one third of claudin-low tumors described by Prat et al. were metaplastic breast carcinomas (MBC) by...
Molecular Pathology and Immunology of Porto University, Ribeirão Preto, Brazil, and Institute of UNIFESP, Brazil, Department of Pathology, School of Medicine, Federal University of São Paulo (UNIFESP), Brazil. EGFR and cytokeratins 5/6. Considering the overlap between factor 2 (HER2), in general positive for epidermal growth factor 1 (EGFR) and cytokeratins 5/6.14 Considering the overlap between HER2-overexpressing (HER2-OE) and non-MBC triple negative tumors, to better establish if there was a specific epithelial component.10,12 Immunohistochemistry is a valuable tool for the diagnosis of MBCs, being in general positive for cytokeratins (at least focally), myoepithelial markers (such as p63) and vimentin.10,12,13 MBC are triple negative tumors [negative for estrogen and progesterone receptors (ER and PgR) and human epidermal growth factor 2 (HER2)], in general positive for epidermal growth factor 1 (EGFR) and cytokeratins 5/6.14 Considering the overlap between these two groups of tumors, the aim of this study was to evaluate, for the first time, CLDNs expression within a series of MBCs, with special attention to the different components within this special type of breast tumors, exploring the described claudin-low features: low CLDNs expression (studying CLDN-1, CLDN-3, CLDN-4 and CLDN-7), EMT phenotype (evaluating E-cadherin and vimentin expression) and CSC-like features (analyzing the well-established ALDH1 and CD44+/CD24− breast cancer stem cell markers). A comparison group of tumors was also used, in order to test CLDNs, as well as CSC and EMT markers, in a group of luminal, HER2-overexpressing (HER2-OE) and non-MBC triple negative tumors, to better establish if there was a specificity of claudin-low phenotype within the MBC group of tumors.

Material and methods

Patient selection

Thirteen patients with MBC were included in this study (Table 1). The tumors were retrieved from the Department of Pathology, School of Medicine, Federal University of São Paulo (UNIFESP), Brazil, Department of Pathology, School of Medicine, University of São Paulo, Ribeirão Preto, Brazil, and Institute of Molecular Pathology and Immunology of Porto University (IPATIMUP), Portugal. The tumors were classified according to the criteria established by The World Health Organization (WHO). Briefly, MBCs where classified as follows: mixed epithelial/mesenchymal (5 cases), squamous cell carcinoma (5 cases) and monophasic spindle cell carcinoma (3 cases). The mixed type included adenocarcinomas with chondroid heterologus tissue and spindles cells. Squamous cell carcinomas were admixed with spindles cells in four cases. Three cases were classified as monophasic spindle cell carcinomas, presenting a pure form of MBC with only spindle cell component. In order to use a comparison group, a series of 463 primary and sporadic invasive breast carcinomas were also included in this study, retrieved consecutively from the Pathology Department, Hospital Xeral-Cíes, Vigo, Spain, diagnosed between 1978 and 1992. The formalin-fixed paraffin-embedded histological sections were reviewed and the diagnoses confirmed, in order to exclude MBCs within this series. Tumors were previously characterized for clinical and pathological features, namely for molecular subtypes based on ER, PgR and HER2 immunohistochemical data: 340 luminal tumors (ER+/PgR−/HER2−), 33 HER2-OE tumors (ER+/PgR+/HER2−) and 90 triple negative tumors (ER−/PgR−/HER2−). This study was conducted under the national regulative law for the handling of biological specimens from tumor banks, the samples being exclusively available for research purposes in retrospective studies.

Immunohistochemistry

To study CLDNs expression in both series, specific antibodies for CLDN-1 [PAB4781, InVitrogen, Carlsbad, CA, USA (Citrate Buffer; 1:800)], CLDN-3 [Z233JM, Invitrogen, Carlsbad, CA, USA (Citrate Buffer; 1:300)], CLDN-4 [Clone Ab15104, Abcam, Cambridge, MA (Citrate Buffer; 1:300)] and CLDN-7 [clone Ab27287, Abcam, Cambridge, MA (Citrate Buffer; 1:400)] were assessed. Specific antibodies for CD44 (clone 156-3C11, Cell Signaling Technology, Danvers, MA (Citrate Buffer; 1:100)), CD24 (clone Ab2-SN3b, NeoMarkers, Fremont, CA (Citrate Buffer; 1:100)), ALDH1 [aldehyde dehydrogenase, clone EP1933Y, Abcam, Cambridge, MA (Citrate Buffer; 1:100)], E-cadherin [clone 24E19, Signalizing Technology, Danvers, MA (Citrate Buffer; 1:100)] and Vimentin [clone V9, Dako, Denmark (Citrate Buffer; 1:150)] were also assessed. The primary antibodies were detected using a secondary antibody with HRP polymer (Cytomation Envision System HRP, DAKO, Carpinteria, CA). For the visualization of the reaction, diaminobenzidine was used as chromogen, according with manufacturer’s instructions.

Membrane CLDNs expression was analyzed by two observers, which registered the intensity and extension of the membrane expression of each CLDN. The intensity of membrane immunostaining was graded as: 0 (negative); 1 (weak); 2 (moderate); 3 (strong). The extension was registered as the percentage of positive cells for CLDNs: 1 (0–10%); 2 (10–25%); 3 (25–50%); 4 (>50%). Then a score was generated by multiplying the intensity by the extension of CLDN immunostaining (scale 0–12). According to the scores, the membrane immunostaining was graded as negative, weak (+) (scores 1, 2 or 3), moderate (++) (scores 4 or 6) and strong (++++) (scores 8, 9 or 12). To explore the presence of the CSC phenotype CD44+/CD24− within MBC, we decided to study CD44 and CD24 expression and to consider a tumor with CSC phenotype when the frequency of CD44+/CD24− cells were more than 10%, as previously described in our and other studies.15–17 The expression of the cancer stem cell marker ALDH1, as well as of the EMT markers E-cadherin and Vimentin, was also evaluated in both tumor series, as previously described by our group.15,18,19

<table>
<thead>
<tr>
<th>MBC</th>
<th>Histological subtype of MBC</th>
<th>Age (years)</th>
<th>Size (cm)</th>
<th>Histological grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mixed epithelial/mesenchymal</td>
<td>39</td>
<td>4.9</td>
<td>3</td>
</tr>
<tr>
<td>II</td>
<td>Mixed epithelial/mesenchymal</td>
<td>71</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td>III</td>
<td>Mixed epithelial/mesenchymal</td>
<td>52</td>
<td>1.6</td>
<td>3</td>
</tr>
<tr>
<td>IV</td>
<td>Mixed epithelial/mesenchymal</td>
<td>33</td>
<td>7.5</td>
<td>3</td>
</tr>
<tr>
<td>V</td>
<td>Mixed epithelial/mesenchymal</td>
<td>50</td>
<td>3.0</td>
<td>3</td>
</tr>
<tr>
<td>VI</td>
<td>Squamous cell carcinoma with spindle cell component</td>
<td>60</td>
<td>3.0</td>
<td>3</td>
</tr>
<tr>
<td>VII</td>
<td>Squamous cell carcinoma with spindle cell component</td>
<td>55</td>
<td>4.2</td>
<td>3</td>
</tr>
<tr>
<td>VIII</td>
<td>Squamous cell carcinoma with spindle cell component</td>
<td>85</td>
<td>12.0</td>
<td>3</td>
</tr>
<tr>
<td>IX</td>
<td>Squamous cell carcinoma with spindle cell component</td>
<td>59</td>
<td>NA</td>
<td>2</td>
</tr>
<tr>
<td>X</td>
<td>Squamous cell carcinoma</td>
<td>54</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>XI</td>
<td>Monophasic spindle cell carcinoma</td>
<td>91</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>XII</td>
<td>Monophasic spindle cell carcinoma</td>
<td>22</td>
<td>11.0</td>
<td>3</td>
</tr>
<tr>
<td>XIII</td>
<td>Monophasic spindle cell carcinoma</td>
<td>76</td>
<td>6.0</td>
<td>3</td>
</tr>
</tbody>
</table>
Results

Searching for the claudin-low phenotype in a series of metaplastic breast carcinomas

The mean age of the patients was 57.5 years (22–91 years) and the size of the tumors ranged from 1.6 to 12.0 cm (mean 5.6 cm) (Table 1). All cases were previously analyzed by immunohistochemistry for ER, PgR and HER2 and considered negative for all these markers (data not shown).

Different types of metaplastic components were present in the representative tumor blocks, including squamous cells, spindle cells and chondroid heterologous elements. The majority of these cases (76.9%, 10/13) presented lymphoid infiltrate.

The majority of CLDN membrane staining in epithelial neoplastic cells was weak (+) or negative, being 76.9% (10/13) low expressing cases for CLDN-1, 84.6% (11/13) for CLDN-3 and CLDN-4, and 92.3% (12/13) for CLDN-7. The spindle cell components and chondroid heterologous elements were always negative for all CLDN members studied (Table 2). In mixed epithelial/mesenchymal and squamous cell carcinomas, CLDNs expression, when present, was only observed in the epithelial component of these tumors.

Interestingly, when we explored the presence of the CSC phenotype CD44+/CD24+/low within MBCs, the majority of the cases were considered CD44+/CD24+/low >10%, and this pattern of immunoeexpression was found in all types of tissue components, including epithelial cells (both glandular and squamous cells), spindle cells and chondroid heterologous tissue. Regarding ALDH1, 30.8% (4/13) showed positive staining: in three cases, the tumor and, in one case, ALDH1 staining was observed in spindle cells.

Overall, monophasic spindle cell carcinoma presented a uniform pattern of expression with low expression of CLDNs and E-cadherin, high levels of vimentin and CD44+/CD24+/low enrichment (Fig. 1). In contrast, tumors presenting two or three components presented a mixed immunophenotype, with the epithelial component expressing CLDNs (although at low levels) and E-cadherin, contrasting with lack of expression for these molecules in the adjacent spindle cell component that was usually vimentin positive (Fig. 2).

The staining of the four CLDNs was predominantly membranous; however, CLDN-4 staining was also found in the cytoplasm and nuclei of MBC cells. In 69.2% (9/13) of the cases, it has been observed mild to moderate CD44+/CD24+/low cytoplasmatic staining, which was mainly found in the epithelial component (Table 2). Concerning aberrant nuclear expression of CD44+/low, it was also found in 69.2% (9/13) of the cases; however, this pattern of staining was found in the different components of MBCs, including squamous cells, spindle cells and chondroid heterologous tissue. In all these cases, membrane staining was negative or weak (+). Remarkably, many cases with nuclear staining also presented cytoplasmic expression of CD44+/low (Table 2).

Searching for the claudin-low phenotype in a series of luminal, HER2-overexpressing and non-metaplastic triple negative breast carcinomas

In order to find out if the claudin-low phenotype was specific for the metaplastic breast carcinomas, we decided to search for this phenotype in a comparison group of breast carcinomas, including

Table 2

<table>
<thead>
<tr>
<th>MBC Histological subtype of MBC</th>
<th>Tumor components</th>
<th>CLDN-1 Memb</th>
<th>CLDN-3 Memb</th>
<th>CLDN-4 Memb</th>
<th>CLDN-7 Memb</th>
<th>CD44+/CD24+/low</th>
<th>ALDH1</th>
<th>Vim</th>
<th>E-cad</th>
<th>L.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Mixed epithelial/mesenchymal</td>
<td>Chondroid heterologous tissue</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II Mixed epithelial/mesenchymal</td>
<td>Spindle cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>III Mixed epithelial/mesenchymal</td>
<td>Adenocarcinoma</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IV Squamous cell carcinoma with spindle cell component</td>
<td>Spindle cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>VII Squamous cell carcinoma with spindle cell component</td>
<td>Squamous carcinoma</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>VIII Squamous cell carcinoma with spindle cell component</td>
<td>Squamous carcinoma</td>
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<td>+</td>
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<tr>
<td>IX Squamous cell carcinoma with spindle cell component</td>
<td>Squamous carcinoma</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
<td>XI Monophasic spindle cell carcinoma</td>
<td>Spindle cells</td>
<td>+</td>
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</tr>
<tr>
<td>XII Monophasic spindle cell carcinoma</td>
<td>Spindle cells</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Memb — Membrane; Cyto — Cytoplasm; Nucl — Nuclei; Vim — Vimentin; E-cad — E-cadherin; L.I. — Lymphoid infiltrate.

a NA — Not available for IHC evaluation.

b (−) — scores 1 or 2; (+) — scores 4 or 6; (+++) — scores 8, 9 or 12.
luminal, HER2-overexpressing and non-MBC triple negative tumors. The expression of CLDNs, CD44 and CD24, ALDH1, Vimentin and E-cadherin were studied in 340 luminal tumors, 33 HER2-OE tumors and 90 triple negative tumors. As can be seen in Fig. 3, only the mesenchymal component, from all the MBCs studied, showed a "pure" claudin-low phenotype, since none of the cases showed the expression of any claudin evaluated; additionally, all the cases were E-cadherin negative and positive for Vimentin, and a high percentage of cases showed the breast CSC (BCSC) phenotype (83.3%). Interestingly, the BCSC phenotype addressed by CD44 and CD24 expression, as well as the ALDH1 expression, were also more frequent in the epithelial component of MBCs (88.9% and 33.3%, respectively), when compared with the frequency found in the other molecular subtypes. Concerning E-cadherin expression, it was possible to see that it was highly prevalent in all groups of breast carcinomas (>90%), being decreased in the epithelial component (66.7%) and completely lost in the mesenchymal component of MBCs. In contrast, Vimentin expression was less frequent in the luminal tumors (8.3%), being this frequency increased in HER2-OE and triple negative tumors (18.2% and 49.4%, respectively), and 100% in the mesenchymal component of MBCs. Finally, concerning claudins expression, CLDN-1 expression was increased in triple negative tumors (12.4%) and more prevalent in the epithelial component of MBCs (33.3%). CLDN-4 was also mostly found in HER2-OE (39.4%) and triple-negative (42.5%) tumors, being also found in the epithelial component of MBCs (33.3%). We did not observe significant variation of the expression of CLDN-3 and CLDN-7 in all groups of tumors, with the exception of the already mentioned absence of all claudins in the mesenchymal component of MBCs.

Discussion

MBCs share unusual morphological characteristics, since part or all tumor cells appear to have undergone transformation to a non-glandular or mesenchymal cell type. The non-glandular component can range from squamous differentiation to diffuse mesenchymal differentiation. This diversity was confirmed by our results, in which we showed different expression patterns for CLDNs and E-cadherin between the distinct morphological components. In biphasic MBCs, we observed expression of CLDNs and E-cadherin in the squamous carcinoma and adenocarcinoma components, in contrast with the chondroid and spindle cell components, where low expression levels of these proteins were found. The monophasic spindle cell carcinoma showed a homogeneous pattern of expression, with negative or low levels of CLDNs and E-cadherin and positivity for vimentin.

In some MBCs, the malignant glands appear to merge continuously with malignant spindle cells. This observation, together with the frequent immunohistochemical expression of keratins within the spindle cell component, suggest that malignant spindle cells are derived from the carcinoma component. In fact, one possible scenario for tumor heterogeneity is that the target cell of transformation can be a multipotent progenitor, with the capacity for epithelial, myoepithelial and/or mesenchymal differentiation. The BCSC phenotype (CD44+CD24-ALDH1-CD24+/low) was found in all MBCs of our cases. This result demonstrate one of the major described characteristics of claudin-low tumors, suggesting that these tumor cells arise from more immature stem or progenitor cells of the normal breast. Regarding ALDH1 pattern of expression, we only found 30.8% (4/13) of positive cases, being more prevalent.

Fig. 1. Monophasic MBC, in which the sarcomatoid component shows a uniform pattern of expression with low expression of CLDNs and E-cad, high level of vimentin, ALDH1 negativity and CD44+CD24-ALDH1- enrichment. CLDN-4 present cytoplasmic and nuclear expression (amplification 200x).
within the epithelial component of the MBCs. In a previous work, our group proposed that ALDH1 can be a marker of progenitor cancer cells, identifying cells with an increased differentiation compared with the ones identified by CD44. These previous findings corroborate the ones found here, in which the epithelial component tends to have more ALDH1 positive cells than the less differentiated mesenchymal element.

Another MBC feature of claudin-low profile is the presence of lymphocyte infiltrate within the tumors, which we found to be present in the majority of the studied cases. Moreover, the low expression of E-cadherin and the presence of sarcomatoid features, such as positivity for vimentin, are consistent with EMT features, also a finding of the claudin-low subgroup of breast tumors.1,26

Remarkably, in our series, the majority of MBCs presented EMT phenotype. In fact, it is emerging that EMT-inducing molecules control the expression of CLDNs, suggesting that these adhesion molecules are the missing link between EMT and the acquisition of the CSC phenotype.27 Indeed, when the mesenchymal component of MBCs was studied in detailed, all the cases showed EMT features and CSC phenotype and negativity for all CLDNs evaluated.

Unexpectedly, we also found mislocalization of CLDN-4 protein in the cytoplasm and nucleus in some MBC cases. This is consistent with the results of others which showed cytoplasmic expression of CLDNs in breast cancer, including for CLDN-1,28-30 CLDN-3,29 CLDN-4,28,29 and CLDN-7.31,32

Fig. 3. Expression pattern of CLDN, E-Cadherin and Vimentin and BCSC markers in Luminal, HER2-OE and Triple-Negative and the distinct components of MBCs. As shown in the figure, the mesenchymal component of MBC present a phenotype similar to the claudin-low tumors, with negative or low expression of CLDN and E-Cadherin, high levels of vimentin and BCSC phenotype.
staining of CLDNs (and for CLDN-4 in particular) was never reported in breast cancer.41-43 The role of CLDNs in the cytoplasm and nuclei of cancer cells is still not well understood. However, D’Souza et al. showed that phosphorylation of CLDN-3 results in a more diffuse staining of this protein in the intercellular junctions, suggesting a redistribution of this protein from TJs to other membrane areas or to the cytoplasm in ovarian cancer cell lines.39 Dhawan et al. observed the expression of CLDN-1 in the cytoplasm and nuclei of a subgroup of human colon cancers and related this phenomenon to APC mutations.40 In another study, Boireau et al. observed delocalization of CLDN-1 and -4 from TJs to the cytoplasm in human bladder tumors and bladder cell line.41 In this work, the authors have shown that hypermethylation of the gene coding sequence appears responsible for CLDN-4 downregulation in advanced tumors, and that variations of methylation may act as a regulatory mechanism for CLDN-4 expression in the bladder, being responsible for the mislocalization of this molecule out of the cell membrane.41 Therefore, the cytoplasmatic and nuclear staining of CLDN-4 found in MBC may suggest a dysfunctional TJ in these cells.

Accumulating data support the view that CLDNs are important markers in breast cancer and can reflect both biochemical and functional changes that are occurring in the normal epithelium.42 In fact, invasive ductal carcinomas have a more cohesive morphology, in which the presence of TJ molecules such as CLDNs, plays an important role. Our results on the control group confirm this data, since E-cadherin and CLDNs were frequently found in these series. On the other hand, in biphasic MBCs, spindle component is composed by isolated neoplastic spindle cells admixed with variable amounts of collagenous stroma, whereas the isolated neoplastic cells inside lacunae are present in the heterologous chondroid tissue. These histopathological characteristics could be the result of TJ loss, including CLDNs proteins. Other tumors composed by dissociated epithelial neoplastic cells showed loss of CLDNs expression, including loss of CLDN-7 expression in 76% of breast invasive lobular carcinomas.43 Negative expression of CLDN-4 in the diffuse pattern of gastric adenocarcinomas31 and reduced expression of CLDN-1, -4 and -7 in undifferentiated thyroid carcinomas.43 Emerging data suggest that tissue- and differentiation stage-specific expression profiles are either modulated or lost, with both up- and down-regulations of specific CLDNs during tumor progression.44

Interestingly, monophasic spindle cell carcinoma showed a uniform pattern of negative/low expression of CLDNs and E-cadherin and expression of vimentin. The morphology of these tumors are similar to those found by Herrchickowitz et al. when first described claudin-low tumors.5 In this study, it was revealed a murine group of spindloid tumors with significant overlap with human claudin-low tumors. This group of murine tumors showed mesenchymal-like features, such as CK8/18 negativity and smooth muscle actin positivity.2 The similarity (histological and protein expression pattern) between spindloid murine tumor and human spindle cell carcinoma led us to hypothesize that these special MBCs are the ones that comprise a “pure” claudin-low profile.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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