



Dissertação

Artigo de investigação

# **Expression distribution of the breast cancer stem cells markers CD44/CD24 within special histological types**

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CD44/CD24 within special histological types**

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## Resumo

**Introdução e objectivo:** O estudo da expressão dos marcadores CD44/CD24 e ALDH1 é o mais frequentemente utilizado para identificar o fenótipo estaminal (ou *stem*) nos carcinomas da mama, tendo a maioria desses estudos sido realizada em amostras de carcinomas ductais invasores. Assim, a prevalência e o significado clínico do fenótipo *stem* nos carcinomas de tipos histológicos especiais, permanecem desconhecidos. Por conseguinte, o objectivo deste estudo é determinar a distribuição dos marcadores *stem* no carcinomas de mama de tipo histológico especial.

**Métodos:** Foi analisada a expressão de CD44, CD24 e ALDH1 em 117 amostras de carcinomas da mama de tipo histológico especial, tendo os resultados obtidos sido comparados com uma série de 466 carcinomas ductais invasores.

**Resultados:** Os tipos histológicos especiais demonstraram uma maior prevalência de células neoplásicas com expressão CD44<sup>+</sup> (78.2% Vs. 51.2%; p<0.001) e maior prevalência do fenótipo CD44<sup>+</sup>/CD24<sup>-/low</sup> (65.5% Vs. 45.3; p<0.001) e CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> (11.5% Vs. 4.8; p=0.043), quando comparados com os carcinomas ductais invasores. Todos os marcadores mostraram diferenças significativas dentro do grupo dos tipos especiais com os carcinomas. Os carcinomas medulares e metaplásicos demonstraram um significativo enriquecimento no fenótipo CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup>.

**Conclusões:** Os tipos histológicos especiais não são homogêneos em relação à expressão dos marcadores de células *stem* cancerígenas diferindo também neste aspecto dos carcinomas ductais invasores. Utilizando o painel de marcadores de células *stem* cancerígenas CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> fomos capazes de, dentro dos tipos especiais, distinguir os carcinomas medulares e metaplásicos, dois tipos histológicos associados a alto grau histológico e fenótipo de tipo basal.

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## Research Article

# Expression distribution of the breast cancer stem cells markers CD44/CD24 within special histological types

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## Abstract

**BACKGROUND AND AIM:** The study of CD44/CD24 and ALDH1 expression is the most consistently used to identify cancer stem cells (CSC) phenotype on breast cancer. Most of these studies were performed using invasive ductal carcinomas (IDCs) samples. It is largely unknown the prevalence and clinical significance of the CSC phenotypes defined by these markers in breast cancer special histological types. Therefore, the aim of this study is to determine the breast CSC markers distribution among special histological types of breast cancer.

**METHODS:** 117 invasive special type breast carcinomas were analysed for the expression of CD44, CD24 and ALDH1, to evaluate their distribution among the distinct special type and the results were compared to a series of 466 IDCs.

**RESULTS:** When comparing with IDC's, special histological subtypes group displayed higher prevalence of CD44<sup>+</sup> cells (78.2% Vs. 51.2; p<0.001) and higher prevalence of the CSC phenotypes CD44<sup>+</sup>/CD24<sup>-/low</sup> (65.5% Vs. 45.3; p<0.001) and CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> (11.5% Vs. 4.8%; p=0.043). All markers displayed significant differences within the special subtypes group with medullary and metaplastic carcinomas displaying a significant enrichment in the CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> phenotype.

**CONCLUSIONS:** Special histological types of breast carcinomas are not homogeneous in CSC markers expression and differ from IDCs. With the use of a panel of CSC markers that defined the CSC phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup>, we were able to within special types distinguish medullary and metaplastic carcinomas, two histological types associated with high grade and basal-like phenotype.

**Key words:** Breast Cancer; Histological special types; CD44/CD24/ALDH1; Cancer Stem Cells

## Introduction

Breast cancer is nowadays a leading cause of cancer death among women and recognized as a complex and heterogeneous disease, comprised of various histological subtypes, with variable clinical presentations and different underlying molecular signatures<sup>1</sup>.

The histological diversity of adenocarcinomas in the breast has long fascinated pathologists. Most invasive breast cancers are classified as invasive ductal carcinoma not otherwise specified (IDC-NOS), whereas about 25% are histologically defined as 'special types'<sup>1,2</sup>, a group that encompasses many morphological distinct subtypes, like medullary, mucinous, papillary, micropapillary, tubular, among others, being the distinction between these subgroups made based on morphological criteria.

Although special types of breast cancer have been shown to be associated with distinct biological features and carry important clinical implications (e.g. patients with tubular carcinomas have survival rates close to normal life expectancy<sup>3</sup>), the use of information on special types has been limited in tailoring the therapy for breast cancer patients. More and more therapy decision-making is governed by a molecular classification of breast cancer, curiously, this classification was derived mainly from the analysis of IDC-NOS samples and therefore it is unknown whether this classification applies to all histological subtypes<sup>4,5</sup>.

Nevertheless, in recent years it has become apparent that the histopathological characteristics of these cancers may be underpinned by distinct arrays of genetic changes, providing direct evidence for genotypic-phenotypic correlations<sup>6</sup> between morphological patterns and molecular changes in breast cancer like t(12;15)(p13;q25) ETV6–NTRK3 fusion gene in secretory carcinomas<sup>7</sup> or the t(6;9)(q22–23; p23–24) MYB–NFIB fusion gene in adenoid cystic carcinomas<sup>8</sup>.

For these reasons, proper pathological evaluation may effectively support and allow more accurate definition of prognosis and treatment choice in niches of patients diagnosed with special types of breast cancer<sup>9</sup>.

Despite our increased knowledge about this disease and combined treatment with surgery, radiotherapy, chemotherapy and “targeted”-therapies, many breast cancer patients will ultimately develop metastatic disease. One theory that could (at least partially) explain treatment failure is the cancer stem cell (CSC) theory. This theory postulates that cancer may be originated and sustained by a small proportion of stem-like cells that display the ability to main tumour growth by self-renewal and differentiation<sup>10</sup> and also display resistance to chemo<sup>11</sup> and radiotherapy<sup>12</sup>.

Specifically regarding breast cancer, many studies have attempted to demonstrate the presence of breast CSC (BCSC) based on cell surface marker profiles being the phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup> the most consistently associated with cells displaying BCSC characteristics<sup>13,14</sup>. The presence of aldehyde dehydrogenase activity (ALDH) has also been associated with CSC characteristics. Breast cancer cases



presenting ALDH1<sup>+</sup> phenotype are more resistant to platinum-based chemotherapy, more aggressive and associated with worse prognosis<sup>15-18</sup>.

Some authors have also investigated the significance of combining both phenotypes and it has been shown that ALDH1 expression can further divide CD44<sup>+</sup>/CD24<sup>-/low</sup> cell population<sup>19</sup> having similar findings been reported by our group in a recent study<sup>20</sup>. In the same study, we also demonstrate that CD44<sup>+</sup>/CD24<sup>-/low</sup> and ALDH1<sup>+</sup> phenotypes are associated with basal-like tumors both *in-vitro* and *in-vivo*<sup>20</sup>.

Based on current knowledge, there is evidence to support the idea that the use of CD44 and CD24 cell surface markers in combination with ALDH1 activity is the most accurate method to identify and isolate CSC-like cells within breast invasive ductal carcinoma (IDC). Regarding special breast cancer morphological subtypes, to our knowledge, only few studies have been conducted exploring the role of CD44 and CD24 in micropapillary carcinoma of the breast<sup>21-23</sup>. Even in a recent study by Park et al. in which several stem cell-related markers have been tested, only ductal carcinoma samples were used<sup>24</sup>. Others have used cohorts of mainly composed of IDC-NOS with only few cases of special subtypes<sup>19,25</sup>. Therefore, the presence of CSC phenotype in the special breast cancer morphological subtypes remains largely unknown.

In the present study, we analyzed the immunohistochemical expression distribution of the main established breast CSC markers, namely CD44, CD24 and ALDH1, in a series of special histological subtypes of breast carcinomas. In addition, we investigated the correlation between the presence of these markers and the available clinicopathological features. Finally, we compared obtained results with a large series of IDC-NOS where the presence of this BCSC had already been investigated by our group<sup>20</sup> in order to determine whether exists significant differences in the prevalence of CSC phenotypes between IDC-NOS and special histological subtypes.

## Material and methods

### Breast tumour samples

Formalin-fixed, paraffin-embedded tissues of 117 invasive special type breast carcinomas were consecutively retrieved from the histopathology files of private Laboratory of Pathology in Campinas, São Paulo, Brazil. This series contained cases of the following special type: 5 lobular classic and 2 pleomorphic, 16 tubular, 26 mucinous, 6 micropapillary, 8 invasive papillary, 4 typical and 16 atypical medullary, 10 metaplastic and 24 apocrine carcinomas. All cases were reviewed on hematoxylin and eosin-stained (H&E) sections by two pathologists (RG and FS).

**TMA construction**

Representative tumour areas were selected on H&E sections and marked on paraffin blocks. Two tissue cores (2mm in diameter) were obtained from each specimen and deposited into a recipient paraffin block using a TMA workstation (TMA builder 20010.02, Histopathology Ltd., Hungary). Fourteen TMA blocks were constructed, each one containing 24 tissue cores, arranged in a 4 x 6 sector. In each TMA block, normal breast and testicular tissue were included as controls. After construction, 2µm tissue sections were cut and adhered to glass slides (Polysine™, Menzel-Glasser, Germany) for the immunohistochemical studies and a H&E-stained section from each TMA block was reviewed in order to confirm the presence of morphological representative areas of the original lesions.

**Immunohistochemistry**

In order to classify all breast cancer tumours molecularly, we evaluated the expression of some commonly used breast cancer biomarkers, namely the hormonal receptors ER and PgR, the proliferation marker Ki67, the tyrosine kinase receptors HER2 and EGFR, CK5 and also P-cadherin. Immunohistochemistry was performed in 3 mm sections. To study CSC markers in this series, specific antibodies for CD44 (clone 156-3C11; Cell Signaling Technology, Danvers, Massachusetts, USA), CD24 (clone Ab2-SN3b; Neomarkers, Fremont, California, USA) and ALDH1 (clone EP1933Y; Abcam, Cambridge, Massachusetts, USA) were used. The primary antibodies were detected using a secondary antibody with horseradish peroxidase polymer (Cytomation Envision System HRP; DAKO, Carpinteria, California, USA), or a biotinylated goat anti-polyvalent as secondary antibody, followed by the streptavidin-peroxidase complex (Thermo Fisher Scientific, Fremont, California, USA), according to the manufacturer's instructions. Both methods used diaminobenzidine as chromogen. Detailed conditions for each antibody can be found in supplementary table S1.

**Immunohistochemistry evaluation**

The expression of the breast cancer biomarkers ER, PgR, HER2, EGFR, CK5 and P-cadherin was evaluated according to the grading systems already described<sup>26</sup>.

In order to compare our results with previously published studies, CD44, CD24 and ALDH1 was evaluated and scored as previously published<sup>20</sup>. Briefly, CD44 and CD24 staining were detected mainly at the membrane of tumour cells and the scoring was considered as follows: 0, 0-10% of positive tumour cells; 1+, 10-25% of positive tumour cells; 2+, 25-50% of positive tumour cells; 3+, more than 50% of positive tumour cells. Cytoplasmic staining was not considered for any of these markers. For CD44, the cases classified as 0 were considered negative, whereas 1+, 2+ and 3+ were established as positive cases. For CD24, the cases were divided into negative/low, when considered 0 or 1+, or in positive cases, when classified as 2+ or 3+. Immunohistochemical staining of ALDH1 was classified as positive when more than 1% of tumour cells showed clear cytoplasmic positivity, as previously described<sup>15,18</sup>.

### Statistical Analysis

Associations between the CD44, CD24, ALDH1 expression and the different molecular subtypes, the clinicopathological parameters or the different molecular markers were assessed using the  $\chi^2$  test or Fisher's exact test when appropriate. Statistical analyses were carried out using SPSS statistics version 20.0 software and a two-tailed significance level of 5% was considered as statistically significant.

## Results

### Association between the expression of CD44, CD24 and ALDH1 with other breast cancer parameters

The expression of CD44, CD24 and ALDH1 was analysed in all breast cancer cases with adequate sample of tumour after TMA's revision.

The expression of CD44 was analysed in 87 cases and 78.2% (68/87) demonstrated clear positive membrane staining. In contrast, membrane CD24 was classified as negative/low in the majority of the cases (81.1%, 73/90). Concerning ALDH1, 13.3% (8/60) were considered positive, showing clear cytoplasmic expression in tumour cells.

When CD44, CD24 and ALDH1 were associated with classic prognostic factors, as well as with other biomarkers studied, CD44 expression was not significantly associated with any of the available variables. In contrast, a significant correlation between CD24 expression and high grade tumours was found, as 58.8% (10/17) of the positive cases were grade III ( $p=0.011$ ), as well as with absence of ER expression and with HER2 overexpression (table I). CD24 was also correlated with the immunohistochemical molecular subtypes, being the majority of luminal carcinomas classified as CD24<sup>-/low</sup> (90.2%; 55/60).

In case of ALDH1 expression, it was significantly associated with high grade tumours ( $p=0.025$ ), ER negativity ( $p=0.010$ ), PgR negativity ( $p=0.022$ ) and with basal marker expression, namely EGFR ( $p=0.014$ ), CK5 ( $p=0.013$ ) and P-cadherin ( $p<0.016$ ). ALDH1 expression was also associated with triple-negative tumours ( $p=0.010$ ).

### CSC phenotype markers and association with breast cancer parameters

To further explore the association between tumours characteristics and the CSC phenotypes, we decided to consider a tumour with CSC phenotype when the frequency of CD44<sup>+</sup>/CD24<sup>-/low</sup> cells were more than 10% as previously described in other studies<sup>14,27</sup>. Similarly to the approach by Raza Ali H *et al.*<sup>19</sup>, and following our previous findings<sup>20</sup> we further defined a putative CSC phenotype by adding the ALDH1 expression in more than 1% of tumour cells condition to the previous phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup> (in more than 10% of tumour cells), thus defining a CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> phenotype. The phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup> was observed in 65.5% (57/87) whereas the phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> was observed in 11.5% (6/52) of the tumours (table II).

When these phenotypes were correlated with pathological variables and biomarkers, in our special subtypes breast carcinoma series, the phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup> was significantly associated with negative HER2 status ( $p=0.018$ ) and high grade ( $p=0.005$ ). As for the CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> phenotype it was correlated with ER and PgR negativity ( $p=0.008$  and  $p=0.009$ , respectively) and with the presence of basal markers, namely EGFR ( $p=0.022$ ) and CK5 ( $p=0.001$ ) (table II).

The CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> phenotype was further associated with triple-negative tumours in comparison with luminal ones ( $p=0.008$ ) (table II).

**Table I** - Associations between the expression of the breast cancer stem cell markers CD44, CD24 and ALDH1 and the classic breast cancer prognostic factors, biological markers and molecular subtypes

	CD44				CD24				ALDH1			
	n	Positive (%)	Negative (%)	p Value	n	Positive (%)	Neg/low (%)	p Value	n	Positive (%)	Negative (%)	p Value
<b>Histological grade</b>	87	68 (78.2)	19 (21.8)	0.092	90	17 (18.9)	73 (81.1)	0.011	60	8 (13.3)	52 (86.7)	0.025
Grade I	33	28 (84.8)	5 (15.2)		33	1 (3.0)	32 (97.0)		24	0 (0.0)	24 (100.0)	
Grade II	17	10 (58.8)	7 (41.2)		18	6 (33.3)	12 (66.7)		14	2 (14.3)	12 (85.7)	
Grade III	37	30 (81.1)	7 (18.9)		39	10 (25.6)	29 (74.4)		22	16 (72.7)	6 (27.3)	
<b>ER</b>	87	68 (78.2)	19 (21.8)	0.615	90	17 (18.9)	73 (81.1)	0.009	60	8 (13.3)	52 (86.7)	0.010 <sup>b</sup>
Positive	60	46 (76.7)	14 (23.3)		61	7 (11.5)	54 (88.5)		41	2 (4.9)	39 (95.1)	
Negative	27	22 (81.1)	5 (18.5)		29	10 (34.5)	19 (65.5)		19	6 (31.6)	13 (68.4)	
<b>PgR</b>	87	68 (78.2)	19 (21.8)	0.667	90	17 (18.9)	73 (81.1)	0.363	59	8 (13.6)	51 (86.4)	0.022 <sup>b</sup>
Positive	45	36 (80.0)	9 (20.0)		46	7 (15.2)	39 (84.8)		31	1 (3.2)	30 (96.8)	
Negative	42	32 (76.2)	10 (23.8)		44	10 (22.7)	34 (77.3)		28	7 (25.0)	21 (75.0)	
<b>HER2</b>	87	68 (78.2)	19 (21.8)	0.364 <sup>a</sup>	90	17 (18.9)	73 (81.1)	0.005 <sup>b</sup>	60	8 (13.3)	52 (86.7)	0.133 <sup>b</sup>
Positive	8	5 (62.5)	3 (37.5)		8	5 (62.5)	3 (37.5)		1	1 (100.0)	0 (0.0)	
Negative	79	63 (79.7)	16 (20.3)		82	12 (14.6)	70 (85.4)		59	7 (11.9)	52 (88.1)	
<b>Ki67</b>	81	62 (76.5)	19 (23.5)	0.925	84	15 (17.9)	69 (82.1)	0.101 <sup>b</sup>	57	7 (12.3)	50 (87.7)	0.660 <sup>b</sup>
Positive	59	45 (76.3)	14 (23.7)		62	14 (22.6)	48 (77.4)		41	6 (14.6)	35 (85.4)	
Negative	22	17 (77.3)	5 (22.7)		22	1 (4.5)	21 (95.5)		16	1 (6.3)	15 (93.8)	
<b>EGFR</b>	86	67 (77.9)	19 (22.1)	0.542 <sup>a</sup>	89	16 (18.0)	73 (82.0)	0.469	59	8 (13.6)	51 (86.4)	0.014 <sup>b</sup>
Positive	20	17 (85.0)	3 (15.0)		20	4 (20.0)	16 (80.0)		14	5 (35.7)	9 (64.3)	
Negative	60	50 (75.8)	16 (24.2)		69	12 (17.4)	57 (82.6)		45	3 (6.7)	42 (93.3)	
<b>CK5</b>	86	67 (77.9)	19 (22.1)	0.439	89	17 (19.1)	72 (80.9)	0.459	60	8 (13.3)	52 (86.7)	0.013 <sup>b</sup>
Positive	29	24 (82.8)	5 (17.2)		30	7 (23.3)	23 (76.7)		20	6 (30.0)	14 (70.0)	
Negative	57	43 (75.4)	14 (24.6)		59	10 (16.9)	49 (83.1)		40	2 (5.0)	38 (95.0)	
<b>P-cadherin</b>	86	67 (77.9)	19 (22.1)	0.795	89	17 (19.1)	72 (80.9)	0.166	59	8 (13.6)	51 (86.4)	0.016 <sup>b</sup>
Positive	52	41 (78.8)	11 (21.2)		55	13 (23.6)	42 (76.4)		35	8 (22.9)	27 (77.1)	
Negative	34	26 (76.5)	8 (23.5)		34	4 (11.8)	30 (30.2)		24	0 (0.0)	24 (100.0)	
<b>Molecular subtypes</b>	87	68 (78.2)	19 (21.8)	0.262	90	17 (18.9)	73 (81.1)	0.004	60	8 (13.3)	52 (86.7)	0.010 <sup>b</sup>
Luminal	60	45 (75.0)	15 (25.0)		60	6 (9.8)	55 (90.2)		41	2 (4.9)	39 (95.1)	
HER2-OE	6	4 (66.7)	2 (33.3)		6	3 (50.0)	3 (50.0)		-	-	-	
Triple-negative	21	19 (90.5)	2 (9.5)		23	8 (34.8)	15 (65.2)		19	6 (31.6)	13 (68.4)	

<sup>a</sup> Percentage of row total

<sup>b</sup> Two-sided Fisher's exact test

### Breast cancer special subtypes and CSC phenotype markers

Our special breast cancer subtypes series was composed of 9 morphological subtypes that were condensed in 8 categories, since classic lobular and lobular pleomorphic were classified as “lobular”. Therefore, our series was composed of: 22.2% mucinous (26/117), 17.1% medullary (20/117), 13.7% tubular (16/117), 20.5% invasive apocrine (24/117), 6.0% lobular (7/117), 8.5% metaplastic (10/117), 5.1% micropapillary (6/117) and 6.8% papillary (8/117) breast invasive carcinomas.

**Table II** - Associations between the expression of the breast cancer stem cell markers phenotypes CD44<sup>+</sup>/CD24<sup>-/low</sup>, CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> the breast cancer prognostic factors, biological markers and molecular subtypes

	CD44/CD24				CSC phenotype (CD44/CD24/ALDH1)			
	n	CD44 <sup>+</sup> /CD24 <sup>-/low</sup> >10% <sup>(a)</sup>	CD44 <sup>+</sup> /CD24 <sup>-/low</sup> <10% <sup>(a)</sup>	p Value	n	Present <sup>(a)</sup>	Absent <sup>(a)</sup>	p Value
<b>Histological grade</b>	87	57 (65.5)	30 (34.5)	0.005	52	6 (11.5)	46 (88.5)	0.064
Grade I	33	27 (81.8)	6 (18.2)		23	0 (0.0)	23 (100.0)	
Grade II	17	6 (35.3)	11 (64.7)		11	2 (18.2)	9 (81.8)	
Grade III	37	24 (64.9)	13 (35.1)		18	4 (22.2)	14 (77.8)	
<b>ER</b>	87	57 (65.5)	30 (34.5)	0.410	52	6 (11.5)	45 (88.5)	0.008 <sup>b</sup>
Positive	60	41 (68.3)	19 (31.7)		36	1 (2.8)	35 (97.2)	
Negative	27	16 (59.3)	11 (40.7)		16	5 (31.3)	11 (68.8)	
<b>PgR</b>	87	57 (65.5)	30 (34.5)	0.493	52	6 (11.5)	46 (88.5)	0.009 <sup>b</sup>
Positive	45	31 (68.9)	14 (31.1)		27	0 (0.0)	27 (100.0)	
Negative	42	26 (61.9)	16 (38.1)		25	6 (24.0)	19 (76.0)	
<b>HER2</b>	87	57 (65.5)	30 (34.5)	0.018 <sup>b</sup>	52	6 (11.5)	46 (88.5)	1.000 <sup>b</sup>
Positive	8	2 (25.0)	6 (75.0)		1	0 (0.0)	1 (100.0)	
Negative	79	55 (69.6)	24 (30.4)		51	6 (11.8)	45 (88.2)	
<b>Ki67</b>	81	53 (65.4)	28 (34.6)	0.399	49	5 (10.2)	44 (89.8)	1.000 <sup>b</sup>
Positive	59	37 (62.7)	22 (37.3)		36	4 (11.1)	32 (88.9)	
Negative	22	16 (72.7)	6 (27.3)		13	1 (7.7)	12 (92.3)	
<b>EGFR</b>	86	57 (66.3)	29 (33.7)	0.688	51	6 (11.8)	45 (88.2)	0.022 <sup>b</sup>
Positive	20	14 (70.0)	6 (30.0)		12	4 (33.3)	8 (66.7)	
Negative	66	43 (65.2)	23 (34.8)		39	2 (5.1)	37 (94.9)	
<b>CK5</b>	86	56 (65.1)	30 (34.9)	0.956	52	6 (11.5)	46 (88.5)	0.001 <sup>b</sup>
Positive	29	19 (65.5)	10 (34.5)		17	6 (35.3)	11 (64.7)	
Negative	57	37 (64.9)	20 (35.1)		35	0 (0.0)	35 (100.0)	
<b>P-cadherin</b>	86	56 (65.1)	30 (34.9)	0.690	52	6 (11.5)	46 (88.5)	0.075 <sup>b</sup>
Positive	52	33 (63.5)	19 (36.5)		33	6 (18.2)	27 (81.8)	
Negative	34	23 (67.6)	11 (32.4)		19	0 (0.0)	19 (100.0)	
<b>Molecular subtypes</b>	87	57 (65.5)	30 (34.5)	0.226	52	6 (11.5)	46 (88.5)	0.008 <sup>b</sup>
Luminal	60	41 (68.3)	19 (31.7)		36	1 (2.8)	35 (97.2)	
HER2-OE	6	2 (33.3)	4 (66.7)		-	-	-	
Triple-negative	21	14 (66.7)	7 (33.3)		16	5 (31.3)	11 (68.8)	

<sup>a</sup> Percentage of row total

<sup>b</sup> Two-sided Fisher's exact test

Between each special subtype, there were significant differences in the prevalence of CD44 ( $p=0.001$ ) and ALDH1 ( $p=0.002$ ) expression, as well as in the prevalence of CD44<sup>+</sup>/CD24<sup>-/low</sup> phenotype ( $p=0.002$ ) and the combined CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> phenotype ( $p=0.042$ ) (table III and IV).

To explore the differences in the prevalence of expression of these markers between specific special subtypes, we compared obtained results with a series previously characterised in our group and composed of 465 IDC-NOS characterised for CD44, CD24 and ALDH1, whose results had already been published<sup>20</sup>. When grouped together, special subtypes (as one group composed of all tumours in this series), 78.2% (68/87) were CD44<sup>+</sup> and 65.5% (57/87) displayed the CSC phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup>, being both results significantly higher than the observed in the IDC-NOS comparison series (both  $p<0.001$ ). And though ALDH1 expression was no different, special subtypes also displayed an enriched population with the phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> ( $p=0.043$ ).

When performing a subgroup analysis by special subtype and comparing to IDC-NOS, several differences were found. Regarding CD44 expression, medullary and tubular carcinomas were subtypes enriched in this marker (both associations with  $p<0.001$ ), while regarding CD24 expression, both invasive apocrine and papillary carcinomas displayed increased expression in this marker (42.9% and 100% Vs. 11.4% in IDC-NOS, with  $p=0.004$  and  $p<0.001$ , respectively) (table III).

**Table III** – Prevalence of the expression of CD44, CD24 and ALDH1 in the breast cancer histological special subtypes

	CD 44			p Value <sup>b</sup>	CD 24			p Value <sup>b</sup>	ALDH1			p Value <sup>b</sup>
	n	Positive (%) <sup>a</sup>	Negative (%) <sup>a</sup>		n	Positive (%) <sup>a</sup>	Negative (%) <sup>a</sup>		n	Positive (%) <sup>a</sup>	Negative (%) <sup>a</sup>	
IDC-NOS <sup>c</sup>	463	237 (51.2)	226 (48.8)		463	53 (11.4)	410 (88.7)		464	33 (7.1)	431 (92.9)	
<b>Special subtype</b>	87	68 (78.2)	19 (21.8)	<0.001	90	17 (18.9)	73 (81.1)	0.052	60	8 (13.3)	52 (86.7)	0.119 <sup>d</sup>
mucinous	21	15 (71.4)	6 (28.6)	0.069	21	2 (9.5)	19 (90.5)	1.000 <sup>d</sup>	19	0 (0.0)	19 (100.0)	0.632 <sup>d</sup>
medullary	15	15 (100.0)	0 (0.0)	<0.001 <sup>d</sup>	15	3 (20.0)	12 (80.0)	0.402 <sup>d</sup>	12	5 (41.7)	7 (58.3)	<0.001 <sup>d</sup>
tubular	14	14 (100.0)	0 (0.0)	<0.001 <sup>d</sup>	14	0 (0.0)	14 (100.0)	0.383 <sup>d</sup>	9	0 (0.0)	9 (100.0)	1.000 <sup>d</sup>
invasive apocrine	13	9 (69.2)	4 (30.8)	0.199	14	6 (42.9)	8 (57.1)	0.004 <sup>d</sup>	-	-	-	
lobular	7	2 (28.6)	5 (71.4)	0.278 <sup>d</sup>	7	2 (28.6)	5 (71.4)	0.192 <sup>d</sup>	5	0 (0.0)	5 (100.0)	1.000 <sup>d</sup>
metaplastic	7	6 (85.7)	1 (14.3)	0.124 <sup>d</sup>	8	2 (25.0)	6 (75.0)	0.237 <sup>d</sup>	9	2 (22.2)	7 (77.8)	0.138 <sup>d</sup>
micropapillary	6	3 (50.0)	3 (50.0)	1.000 <sup>d</sup>	6	2 (33.3)	4 (66.7)	0.149 <sup>d</sup>	1	1 (100.0)	0 (0.0)	0.073 <sup>d</sup>
papillary	4	4 (100.0)	0 (0.0)	0.124 <sup>d</sup>	5	5 (100.0)	0 (0.0)	<0.001 <sup>d</sup>	5	5 (100.0)	0 (0.0)	<0.001 <sup>d</sup>

For comparison of CD44 expression between special subtypes: p value = 0.001

For comparison of CD24 expression between special subtypes: p value = 0.090

For comparison of ALDH1 expression between special subtypes: p value = 0,002

<sup>a</sup> percentage of raw total

<sup>b</sup> for comparison with IDC-NOS

<sup>c</sup> as previously published by Ricardo S *et al.*<sup>20</sup>

<sup>d</sup> two-sided Fisher's exact test

**Table IV** – Prevalence of the expression of CSC phenotype CD44/CD24 and CD44/CD24/ALDH1 in the breast cancer histological special subtypes

	CD44/CD24			p Value <sup>b</sup>	CSC phenotype (CD44/CD24/ALDH1)			p Value <sup>b</sup>
	n	CD44 <sup>+</sup> /CD24 <sup>-/low</sup> >10% <sup>(a)</sup>	CD44 <sup>+</sup> /CD24 <sup>-/low</sup> <10% <sup>(a)</sup>		n	Positive <sup>(a)</sup>	Negative <sup>(a)</sup>	
IDC-NOS <sup>c</sup>	461	209 (45.3)	252 (54.7)		459	22 (4.8)	437 (95.2)	
<b>Special subtype</b>	87	57 (65.5)	30 (34.5)	<0.001	52	6 (11.5)	46 (88.5)	0.043
mucinous	21	13 (61.9)	8 (38.1)	0.136	17	0 (0.0)	17 (100.0)	1.000 <sup>d</sup>
medullary	15	12 (80.0)	3 (20.0)	0.008	11	4 (36.4)	7 (63.6)	0.002 <sup>d</sup>
tubular invasive apocrine	14	14 (100.0)	0 (0.0)	<0.001 <sup>d</sup>	10	0 (0.0)	10 (100.0)	1.000 <sup>d</sup>
lobular	13	5 (38.5)	8 (61.5)	0.623	-	-	-	-
lobular	7	2 (33.3)	4 (66.7)	0.690 <sup>d</sup>	5	0 (0.0)	5 (100.0)	1.000 <sup>d</sup>
metaplastic	7	5 (71.4)	2 (28.6)	0.255 <sup>d</sup>	7	2 (28.6)	5 (71.4)	0.046 <sup>d</sup>
micropapillary	6	2 (33.3)	4 (66.7)	0.694 <sup>d</sup>	1	0 (0.0)	1 (100.0)	1.000 <sup>d</sup>
papillary	4	4 (100.0)	0 (0.0)	0.043 <sup>d</sup>	1	0 (0.0)	1 (100.0)	1.000 <sup>d</sup>

For comparison of CD44/CD24 expression between special subtypes, p value =0.002

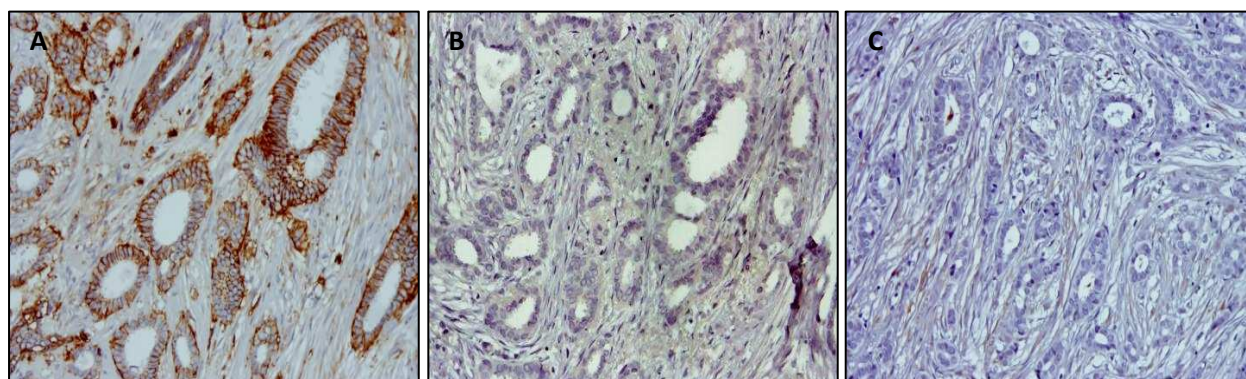
For comparison of CSC expression between special subtypes p value =0.042

<sup>a</sup> percentage of row total

<sup>b</sup> for comparison with IDC-NOS

<sup>c</sup> as previously published by Ricardo S *et al.*<sup>20</sup>

<sup>d</sup> two-sided Fisher's exact test



**Fig. 1.** Immunohistochemical staining of invasive tubular carcinoma for CD44, CD24 and ALDH1. (A) Tubular carcinoma is positive for CD44, in more than 50% of neoplastic cells. (B) In contrast, no expression of CD24 is detected. (C) Similarly, no ALDH1 expression is detected in tubular carcinoma cells.

Concerning ALDH1 expression and the CSC phenotypes, all tubular (14/14) and papillary (4/4) and 80% (12/15) of medullary carcinomas displayed the CD44<sup>+</sup>/CD24<sup>-/low</sup> phenotype. In all these three subtypes, a statistical significant increase was found to IDC-NOS (table 4). Interestingly, only papillary and medullary carcinomas displayed a significant higher proportion of ALDH1 expression.

Finally, the CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> CSC phenotype was observed in 36.4% (4/11) of medullary and 28.6% (2/7) of metaplastic carcinomas, both special types displaying a statistically significant increase (p=0.002 and p=0.046, respectively) over the 4.8% (22/459) prevalence of this CSC phenotype in the “control” IDC-NOS series (table IV).

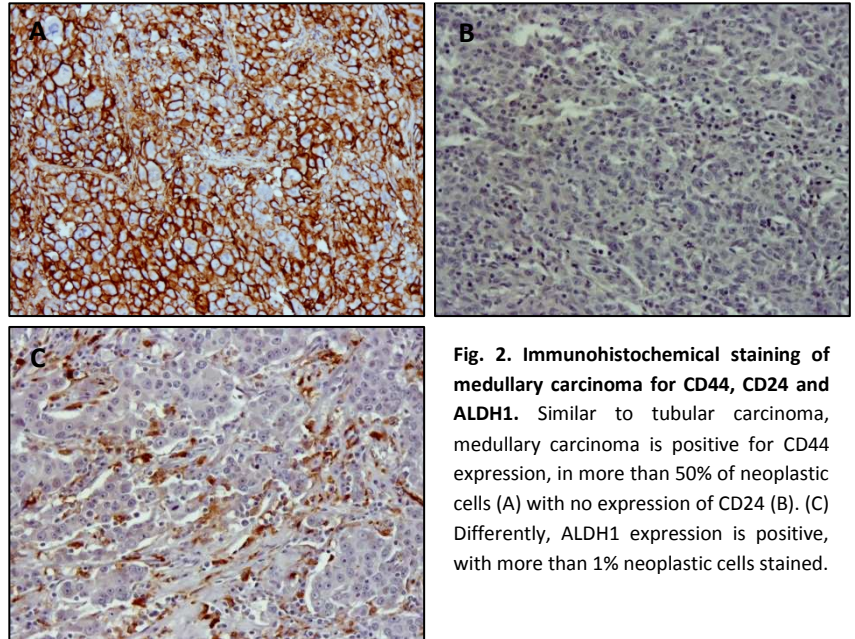
## Discussion

Breast cancer is a heterogeneous disease consisting of a growing number of biologically distinct subtypes with a heterogeneity reflected not only by receptor expression status but also by diverse histological subtypes, as well as distinct biological behavior, response to therapy and disease outcome<sup>1</sup>.

The present study was design at evaluating possible heterogeneity in CSC markers according to

histological special subtypes. To our knowledge, only two studies specifically investigating some of CSC markers and histological special subtypes have been published. In one these studies only the expression of CD44 is evaluated in micropapillary carcinomas and compared to tubular carcinomas<sup>21</sup> while in the other, although it was investigated the immunophenotype  $CD44^+/CD24^{-/low}$  and results compared to a “control” series of IDC-NOS, again, only micropapillary carcinomas were reported<sup>22</sup>. Therefore, our study is the first to address the question of CD44, CD24 and ALDH1 CSC markers prevalence, relation to pathological features and a series of biomarkers in a cohort with tumors of several different histological subtypes. Furthermore, it compares each special subtype represented with previously published data by our group of a large cohort of IDC-NOS to explore possible enrichment of these markers in specific histological special subtypes.

Regarding specific markers, the CD44 CSC marker is commonly expressed among primary breast carcinomas, whereas expression of CD24 and ALDH1 occurs in a minority of cases<sup>19,20,22,25</sup>. Indeed in our series, when considering all tumors, although special subtypes displayed similar prevalence to the IDC-NOS in CD24 and ALDH1 expression, CD44 was more commonly expressed. When combined CSC immunophenotypes were assessed, we found in the special subtypes group an increase in the prevalence of expression of both  $CD44^+/CD24^{-/low}$  phenotype and in the  $CD44^+/CD24^{-/low}/ALDH1^+$  phenotype. These results strongly contrast with a previous report in which all 9 tumors samples used expressed the CSC phenotype  $CD44^+/CD24^{-/low}$ <sup>28</sup>. Since then, other groups have reported similar prevalence of  $CD44^+/CD24^{-/low}$  tumour cells to the one observed in the present study<sup>22,25,27</sup>, clearly demonstrating that not all breast cancers display  $CD44^+/CD24^{-/low}$ . One fact that could account for this discrepancy is that in Al-Hajj *et al.* study, the authors used samples from 8 metastasis and only 1 primary tumour<sup>28</sup>.



**Fig. 2. Immunohistochemical staining of medullary carcinoma for CD44, CD24 and ALDH1.** Similar to tubular carcinoma, medullary carcinoma is positive for CD44 expression, in more than 50% of neoplastic cells (A) with no expression of CD24 (B). (C) Differently, ALDH1 expression is positive, with more than 1% neoplastic cells stained.



To correlate markers expression with available pathological variables and biomarkers, all tumors in the cohort were considered as a “special subtype” group. In the previous study by our group, CD44 was significantly expressed in basal-like tumours and aggressive basal-like cell lines<sup>20</sup>. It has already been demonstrated that CD44<sup>+</sup> cells show a mesenchymal stem cell-like profile, enriched for genes involved in cell motility, proliferation and angiogenesis<sup>29</sup>. CD44 expression has also been inversely associated with lymph node metastasis<sup>30</sup>. However in this study we fail to reach significance for differences in prevalence of CD44 expression for any of the variables studied, namely grade, hormone receptor status, HER2 and EGFR receptor status, Ck5, P-cadherin, ki-67 proliferation index and molecular subtype.

Differently, in case of CD24 expression, we found an association with negativity for ER and Her2 expression, as well as higher prevalence of expression in grade III tumors. In previous studies which reported CD24, results were contradictory. While in some no associations were found<sup>20,22</sup> others have associated CD24 expression with higher histological grade<sup>31</sup> and unfavorable prognosis<sup>32</sup>. These contradictory results could, at least partially, be explained by the distinct grading systems used to classify CD24 immunohistochemical results<sup>25,27,31,32</sup>, fact that certainly affects the results concerning both the identification and the prognostic value of this marker.

In our study, ALDH1 expression was significantly associated with high grade tumors, ER negativity, PgR negativity, triple-negative tumors and with basal marker expression, namely EGFR, CK5 and P-cadherin. Similar associations with ALDH1 for IDC-NOS were already described in studies with similar methodological approaches by our group<sup>20</sup> and other authors<sup>19</sup>.

For the CSC phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup>, in our special subtypes series, the presence of this phenotype was only associated with HER2 negative status and higher grade tumors. In the study by Honeth *et al.* in a cohort predominantly composed by IDC-NOS, this CSC phenotype was also correlated with low/negative HER2 expression. In the same study this phenotype was further associated with CK5, CK14 and EGFR expression<sup>25</sup>, and when the authors used the 10% cut-off for tumor cells CD44<sup>+</sup>/CD24<sup>-/low</sup> (the same performed in the present study), they further associated this phenotype with ER negativity. In a similar study, the presence of the CSC phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup> was over associated with negative HER2, both in ER positive and ER negative tumors, and a high Ki67 labelling index<sup>19</sup>. In the same study this phenotype was not associated with survival, as we and others have also already demonstrated<sup>20,25</sup>.

Regarding the CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> phenotype, similar results to the ALDH1 expression were found. In case of this CSC phenotype we found associations with both ER and PgR negativity, triple-negative tumors and also with the presence of basal markers, namely EGFR and CK5. Similarly the CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup> phenotype have been associated to worst prognosis in a subgroup of ER negative tumors, in contrast with the phenotype defined only by CD44<sup>+</sup>/CD24<sup>-/low</sup> expression<sup>19</sup>.

When we performed the analysis by histological special type, both medullary and tubular carcinomas were subtypes enriched for CD44 expression. Although this might seem an unexpected result for tubular carcinomas, since these tumors are traditionally described as low grade and well differentiated, an analogous observation regarding high prevalence of CD44 expression in tubular carcinomas has been briefly reported in a study comparing CD44 expression in tubular carcinomas with IMPCs<sup>21</sup>. In that study,

the authors report that 96% of tubular carcinomas expressed CD44 in contrast to only 61% of IMPC. In the same study, CD44 expression was associated with lymph-node metastasis<sup>21</sup>. Due to these findings, in the same article, it is suggested that the loss of CD44s may contribute to reduced cell-cell and cell-basement membrane adhesion, facilitating detachment of tumour cells from primary sites and penetration into lymph-vascular spaces<sup>21</sup>. Nevertheless, the role of CD44 in cancer progression remains largely unknown.

In case of CD24 expression, no differences between special types were found and contrary to a previous report of an increased prevalence of CD24 expression in IMPC<sup>22</sup>, in our study and in comparison with IDC-NOS, only invasive apocrine and papillary carcinomas displayed a significantly increased expression of this marker. Again, although CD24 is known to be involved in cell-cell adhesion and might have an important role in the metastatic process<sup>33</sup>, the significance of its expression remains controversial with some studies associating it with worse prognosis<sup>27</sup> while others do not<sup>14</sup>.

In our series, tubular, papillary and medullary carcinomas were associated with higher prevalence of CD44<sup>+</sup>/CD24<sup>-/low</sup> CSC phenotype. Interestingly some reports mentioned an increased prevalence of this phenotype in medullary carcinomas<sup>25</sup> and in grade I tumours<sup>19</sup>, like the tubular carcinomas. Nevertheless in our results we could not confirm the reported enrichment of the CD44<sup>+</sup>/CD24<sup>-/low</sup> phenotype in IMPC<sup>22</sup>.

When investigating the expression of ALDH1 in special subtypes, only papillary and medullary carcinomas retained ALDH1 expression, with none of tubular carcinomas expressing it. Therefore, in case the combined CSC phenotype CD44<sup>+</sup>/CD24<sup>-/low</sup>/ALDH1<sup>+</sup>, only medullary and metaplastic carcinomas demonstrated significant increase in the prevalence of this phenotype over IDC-NOS. Interestingly these are two histological subtypes associated with high histological grade and basal-like phenotype. Based on these findings we suggest that in case of special subtypes, possibly due to their heterogeneity as a group and simultaneous homogeneity of each special subtype, a combination of several markers is essential for the identification of tumours that possibly exhibit characteristics traditionally associated with CSC.

Nonetheless, our study has some potential limitations. First, although our series contained several histological subtypes, not all histological subtypes were represented nor its weight on the series was adjusted for known prevalence of each of these subtypes. Another limitation when comparing our results to other groups is that for the CSC markers used there is no consensus on their evaluation, with different groups reporting different grading criteria and cut-offs. Nevertheless, in this study, and as already mentioned, we opted to use same methodology as previously used by our group for possible comparisons with the previous published IDC-NOS cohort data<sup>20</sup>.

Other possible limitations of our study could be the assumption of the use of IHC to identify CSC markers and the use of TMAs to detect a subpopulation of cells of reputed scarcity; though there are now a number of studies that used the same methodologies with robust results<sup>19,20,22,25</sup> and therefore we do not consider the methodology used (TMAs and IHC) as relevant limitations.

In summary, our analyses should be considered exploratory and it is our conviction that further validation studies in independent histological special subtypes cohorts are necessary before definitive

conclusions can be drawn. Nevertheless in this study by demonstrating that the prevalence of CSC markers is not homogeneous amongst breast cancer histological types and considering that CD44<sup>+</sup> and CD24<sup>+</sup> cells might represent defined cell populations with distinct genetic profiles<sup>29</sup>, we provide further evidence that the several special subtypes are distinguished entities from IDC-NOS also in CSC markers expression, fact that should be taken into account in future studies investigating the clinical and pathological relevance of CSC markers in breast cancer. Moreover, with our results, we provide additional data that supports future use of use a panel of CSC markers, to identify breast CSC in order to eventually better identify them and effectively translate knowledge of breast CSCs into clinical benefit.

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## Support Material

**Supplementary Table 1 - Antibodies used in the immunohistochemistry study**

Molecular marker	Source	Clone	Dilution	incubation	Antigen retrieval	Detection reagent
<b>ER</b>	Neomarkers	SP1	1:100	1 hr RT	Citrate buffer, pH 6 30 min at 98°C	HRP polymer
<b>HER-2</b>	Neomarkers	SP3	1:80	30 min RT	Citrate buffer, pH 6 30 min at 98°C	SABC
<b>PgR</b>	Novocastra	1A6	1:40	1 hr RT	Citrate buffer, pH 6 30 min at 98°C	HRP polymer
<b>P-cadherin</b>	BD Transduction	56	1:50	1 hr RT	EDTA, pH 8 30 min at 98°C	HRP polymer
<b>CK5</b>	Neomarkers	XM26	1:50	1 hr RT	EDTA, pH 8 30 min at 98°C	SABC
<b>Ki-67</b>	Neomarkers	SP6	1:200	1 hr RT	Citrate buffer, pH 6 30 min at 98°C	HRP polymer
<b>EGFR</b>	Zymed	31G7	1:100	1 hr RT	Pepsin 30 min at 37°C	SABC
<b>CD44</b>	Cell signaling	156-3C11	1:100	30 min RT	Citrate buffer, pH 6 30 min at 98°C	SABC
<b>CD24</b>	Neomarkers	SN3b	1:100	1 hr RT	Citrate buffer, pH 6 30 min at 98°C	HRP polymer
<b>ALDH1</b>	Abcam	EP1933Y	1:100	1 hr RT	Citrate buffer, pH 6 30 min at 98°C	HRP polymer

RT – Room Temperature  
 HRP – Horseradish peroxidase  
 SABC – Streptavidin-biotin complex